Assimilatory and antimicrobial functions of vitamin-binding proteins

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ASSIMILATORY AND ANTIMICROBIAL FUNCTIONS OF VITAMIN-BINDING PROTEINS

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Summary

Vitamins are a group of biochemically and metabolically diverse essential micronutrients required for health, homeostasis, and development. The majority of vitamins cannot be endogenously synthesised sufficiently to satisfy physiological requirement, and therefore must be dietary sourced. Transit of vitamin metabolites through the circulation is facilitated by a number of transport proteins. Proteomic analyses have indicated three of these transport proteins; vitamin D-binding protein, haptocorrin (vitamin B_{12}-binding protein), and retinol-binding protein (vitamin A-binding protein) are constituents of a number of exocrine secretions, including saliva. The aim of this study was to determine whether salivary vitamin-binding proteins have assimilatory functions in respect to their corresponding vitamin ligands, or if they support oral homeostasis.

The relationships between salivary and serum vitamin-binding protein concentrations with commonly employed circulatory markers of systemic vitamin status were determined to assess their suitability as non-invasive biomarkers of vitamins status. Studies were undertaken to define the origin of vitamin-binding proteins in the oral cavity, which may relate to their function.

Vitamin deficiencies have been reported in the common inflammatory disorder; periodontal disease. In this study, longitudinally paired saliva and serum samples from a periodontal disease cohort were assessed before and after treatment to determine if hypovitaminosis was a consequence of disrupted salivary vitamin-binding protein function. Interactions between haptocorrin, and the periodontal disease-associated pathobiont; *Porphyromonas gingivalis,* were experimentally considered.

The findings of this thesis indicate the vitamin-binding proteins studied originated from salivary glands and their outputs increased with mastication. Upregulation of salivary flow (as would occur during the oral processing of food) demonstrated a greater output of salivary vitamin-binding proteins compared to resting flow, thus potentially exerting an assimilatory function. Vitamin-binding proteins were however, present in saliva at resting flow, suggesting they may contribute to the maintenance of oral homeostasis. Data presented in this thesis suggest the vitamin B_{12}-binding protein; haptocorrin, exerted a bacteriostatic effect on *P. gingivalis* by sequestering physiologically active analogues of vitamin B_{12}.

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Abbreviations

1,25-$(OH)_2$-D	1,25-dihydroxyvitamin D
25-OH-D	25-hyydroxyvitamin D
AMN	Amnionless
BBM	Boarder border membrane
BCA	Bicinchoninic acid assay
BHI	Brain heart infusion
C3	Compliment component 3
C5a	Complement component 5a
CAL	Clinical attachment loss
CD36	Cluster determinant 36
CMIA	Chemiluminescent microparticle immunoassay
CUB	Cubilin
DBP	Vitamin D-binding protein
DNA	Deoxyribose nucleic acid
DTT	1,4-Dithiothreitol
EGF	Epidermal growth factor
ELISA	Enzyme-linked immunosorbent assay
FAA	Fastidious anaerobic agar
GCF	Gingival crevicular fluid
GI	Gastrointestinal
Hc	Haptocorrin
Hcy	Homocysteine
HPLC	High performance liquid chromatography
HRP	Horseradish peroxidase
IκB-α	I kappa B alpha
IF	Intrinsic factor
Kgp	Lysine gingipain (Lys-X)
LDS	Lithium dodecyl sulphate
LPS	Lipopolysaccharide
MMA	Methylmalonic acid
MMP	Matrix metalloproteases
MRP1	Multi-drug resistant protein 1
MWS	Molecular weight standard
NCP1L1	Niemann-Pick C1-Like 1
NDNS	National Diet and Nutrition Survey

NF-kB	Nuclear factor-kappa B
NHANES	National Health and Nutrition Examination Survey
OS	Oxidative stress
PD	Periodontal disease
PD	Pocket depth
PKA	Protein kinase A
PTH	Parathyroid hormone
RAR	Retinoic acid receptor
RAREs	Retinoic acid response elements
RBP	Retinol-binding protein (RBP4)
RLU	Relative light unit
RpgA	Arginine gingipain A (Arg-X)
RpgB	Arginine gingipain B (Arg-X)
RXR	Retinoid X receptor
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SEM	Standard error of the mean
sIgA	Secretory immunoglobulin A
SR-B1	Scavenger receptor-class B type 1
STRA6	Stimulated by retinoic acid-6
TBST	Tris buffered saline with $tween_{20}$
TCN-I	Transcobalamin-I (Haptocorrin)
TCN-II	Transcobalamin-II
TCN-II R	Transcobalamin-II-receptor
THF	Tetrahydrofolate
TIMPS	Tissue inhibitors of metalloproteases
UVB	Ultra-violet B
VDR	Vitamin D-receptor
VEGF	Vascular endothelial growth factor
WMS	Whole mouth saliva
WMS(NP)	Whole mouth saliva (excluding parotid)

Chapter 1 Introduction

1.1 Vitamin theory and classification

1.1.1 A brief history of vitamins

Vitamins are a biochemically diverse group of essential micronutrients required to support metabolic homeostasis. For the majority, vitamins cannot be synthesised de novo by humans; therefore, must be exogenously sourced. The term *vitamin* derives from *vitamine* or *vital-amine* which was coined in 1912 by Polish biochemist Casimir Funk who isolated a group of micronutrients found to be essential for life and assumed them to be amines, this was later found to be incorrect and subsequently the “e” was dropped (2). The field of nutrition research in the nineteenth century was centred around the opinion that the essential components of nutrition were: proteins, carbohydrates, fats, and minerals. Upon the advent of germ theory, numerous aetiological organisms for human diseases were being identified, this highlighted a number of prominent diseases such as: pellagra, rickets, beriberi, and scurvy; the causes of which remained elusive. Such diseases would later be attributed to nutritional deficits. The retrospectively named “vitamin theory” was presented in 1906 by British biochemist Frederick Gowland Hopkins, while working at Guy’s Hospital in London (3). He proposed that optimal health could not be sustained on the current dogma of nutritional research and suggested there were other key components of the diet which were required for life. The bulk of research into vitamins was undertaken between the early nineteenth to mid twentieth centuries.

1.1.2 Vitamin classification

Vitamins are typically classified by their solubility. With the lipid-soluble group comprising vitamins; A, D, E, and K; and the water-soluble group consisting of the B-complex (vitamins; B_1, B_2, B_3, B_5, B_6, B_7, B_9, and B_{12}); and vitamin C (4, 5). Although grouped by solubility; each vitamin is structurally distinct, and therefore the vitamins facilitate a diverse number of metabolic roles. Each vitamin title is typically used in reference to a group of structurally and metabolically homologous compounds.

1.1.3 Defining vitamin assimilation

Vitamin assimilation refers to the processes required to sustain the vitamin requirement of cells. Pathways typically involve oral and gastric processing of foods, absorption in the gastrointestinal tract, biochemical processing, which often occurs in the liver, and circulatory transit and cellular uptake.

1.2 Vitamin D

1.2.1 Metabolism and physiology

Vitamin D is the collective name given to a family of steroids, the most physiologically important being vitamin D_2 (ergocalciferol), and vitamin D_3 (cholecalciferol) (Figure 1-1). Vitamin D is relatively sparse in the diet. Ergocalciferol is synthesised largely by fungi, from the UVB irradiation of ergosterol, though some data indicates this metabolite is less effective at improving vitamin status than cholecalciferol (6). Cholecalciferol is present in some animal products such as eggs, oily fish, and some meat products (7). Unlike the other vitamins, vitamin D_3 can by dermally synthesised *de novo* by humans with ultraviolet-B (UVB) dependent conversion of 7-dehydrocholesterol to previtamin D_3, which is then rapidly converted to cholecalciferol in a temperature-dependent reaction (8). The endogenous synthesis of vitamin D is dependent on a number of important factors, including exposure to the critical UVB wavelengths between 290-320nm, with the optimum range of 295-297nm (9, 10). Exposure to such wavelengths is highly dependent on geographical location with extremes of longitude, or seasonality greatly influencing vitamin D status (11, 12). The protective effect of dermal melanin against damage from solar radiation also reduces synthesis of vitamin D, therefore the degree of skin pigmentation can have implications for vitamin D status (13). Furthermore, the use of sun screens, and clothing choice can all impact endogenous synthesis of vitamin D (14).

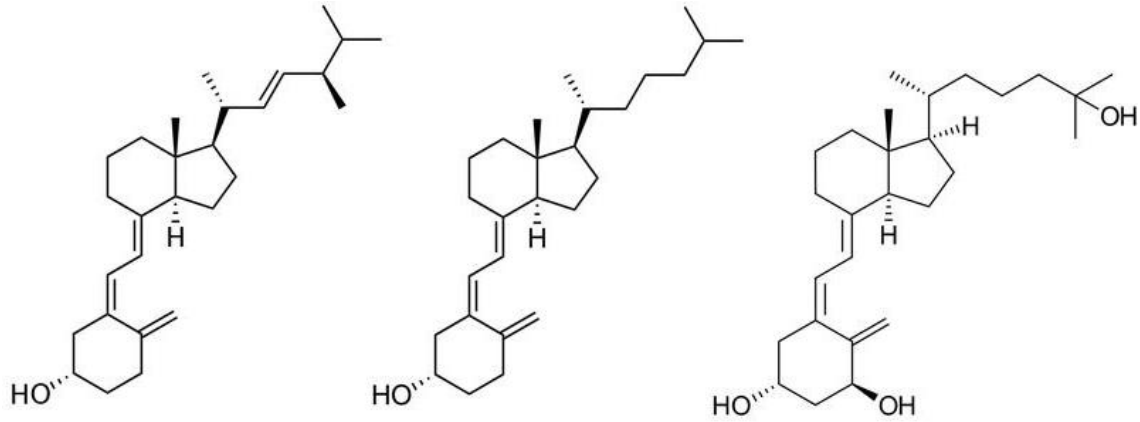

Figure 1-1 Chemical structures of vitamin D metabolites
(Left) ergocalciferol, (centre) cholecalciferol, (right) calcitriol

Vitamin $D_{2/3}$ are biologically inert metabolites and must undertake two hydroxylation steps to become active, firstly 25-hydroxyvitamin D (25-OH-D) is generated in the liver by 25-hydroxylase, the second hydroxylation step occurs in renal tissue by 1α-hydroxylase and generates the hormonally active vitamin D metabolite; 1,25-dihydroxyvitamin D (1,25-$(OH)_2$-D) (calcitriol). Interestingly 1α-hydroxylase activity has been identified in a number of extra-renal tissues including the gingival, suggesting

localised effects of vitamin D (15). The second hydroxylation of 25-OH-D to 1,25-$(OH)_2$-D is regulated by a negative feedback loop, responsive to circulatory concentrations of 1,25-$(OH)_2$-D. 25-OH-D has a significantly longer circulatory half-life than 1,25-$(OH)_2$-D and consequently is a more stable indicator of vitamin D status (16). Currently there is no international consensus on optimal levels of vitamin D, though it is generally accepted that a serum concentration of 25-OH-D <50nmol/L is characteristic of deficiency (17).

The physiologically active metabolite of vitamin D; 1,25-$(OH)_2$-D, was initially identified as being an essential factor for mineral homeostasis. Acting on intestinal, renal, and skeletal tissues, vitamin D acts with parathyroid hormone (PTH) to regulate circulatory calcium and phosphorus concentrations (18). In the late 1960s, Haussler and Norman first described a nuclear receptor for which 1,25-dihydroxyvitamin D was the ligand, this discovery sparked a new interest in vitamins D, and indicated functions beyond the hormonal regulation of calcium (19). Expression of the vitamin D-receptor (VDR) has subsequently been identified in a large number of tissues including those of the digestive-, respiratory-, and immune systems amongst others, and mechanisms describing the supportive role of vitamin D to physiology are numerous (20, 21).

1.2.2 Assimilation

Considering the variability of exposure to sufficient amounts of UVB radiation, for some populations around the globe, dietary vitamin D is particularly important (22). Being a group of lipid-soluble steroids, vitamin D is thought to follow the same absorption processes as lipids. Vitamin D is released from the food matrix with oral and gastric processing of food and incorporated into mixed micelles in the stomach and duodenum (23). Animal model studies indicate that at pharmacological doses vitamin D absorption occurs in a passive manner (24). However, more recent studies suggest that at dietary concentrations, vitamin D uptake is facilitated by a number of steroid receptors including scavenger receptor-class B type 1 (SR-BI), cluster determinant 36 (CD36), and Niemann-Pick C1-Like 1 (NPC1L1). These three receptors are expressed in the brush border membrane of the intestinal tract, and through the use of tissue culture and ex-vivo animal experiments have been demonstrated to facilitate vitamin D uptake by enterocytes (25, 26). Vitamin D is incorporated into chylomicrons (lipoprotein particles consisting of triglycerides, phospholipids, cholesterol, and proteins) within the enterocyte and released into the lymphatic system by exocytosis, chylomicrons subsequently transfer to the circulation from which they are absorbed by the liver (27). In the circulation, the majority of 25-OH-D and 1,25-$(OH)_2$-D is bound to the vitamin D-binding protein DBP,

and to a lesser extent albumin (28, 29). Binding of the lipid soluble vitamin D metabolites to DBP facilitates their transport in the aqueous phase of plasma.

1.2.3 Vitamin D-binding protein

Vitamin D-binding protein (DBP), also referred to as group-specific component (Gc-globulin), is a multifunctional 58kDa globular glycoprotein which shares structural homology to the albumin protein family (30). DBP is capable of binding vitamin D and a range of metabolites including 25-OH-D and 1,25-$(OH)_2$-D. Expression of circulatory DBP is predominantly undertaken by hepatic parenchymal tissue (31, 32). Vitamin D-DBP complexes are taken up by cells in a receptor-mediated- endocytic process (33). In brief, complexes associate with low-density receptor-related protein 2 (LRP2/ megalin) and cubilin (CUBN) which facilitates uptake (34). Megalin and cubilin are responsible for the renal resorption of many complexes including Vitamin D-DBP which can be lost during the glomerular filtration of blood (33, 35, 36).

It has been reported that as much as 98% of vitamin D-binding sites of circulatory DBP remain unoccupied with typical vitamin D status (37). This high degree of under-saturation implies an alternative function to vitamin D transport. In addition to a vitamin D-binding domain, DBP is also able to bind G-actin monomers (38). Other functions of DBP include enhancing the chemotactic effect of the complement component 5a (C5a), which enriches infiltration of neutrophils and monocytes (39, 40). A number of studies have also shown that the sequential deglycosylation of DBP by β-galactosidase and sialidase secreted from immune cells; generates a potent macrophage activating factor (MAF) which upregulates phagocytic activity (41).

1.2.4 Epidemiology and manifestations of hypovitaminosis D

Clinical assessment of vitamin D to categorise vitamin D status is typically based on circulatory 25-OH-D concentration. There remains a great deal of debate relating to what concentration constitutes a ‘healthy’ level (42, 43). Guidelines are typically based on the findings of large scale population analyses, which use bone mineral density and fracture rate as symptoms of insufficient concentrations which can lead to deficiency (43). Estimates suggest as much as 1 billion people globally are deficient in vitamin D ([25-OH-D] <50nmol/L) with rickets and osteomalacia maintaining high prevalence around the world (44). Due to the number of factors which can influence endogenous synthesis of vitamin D, a large proportion of the global population are reliant on dietary or supplementary sources of vitamin D which can also vary greatly with the country of residence (45). A number of studies utilising data from the rolling National Diet and

Nutrition Survey (NDNS) which is undertaken annually in the United Kingdom, has highlighted certain fractions of the population are particularly at risk of deficiency. A prevalence of 25-OH-D (<25nmol/L) was observed in most age groups at 5-20%, and as high as 20-40% in young men and women over the age of 85. These values increased to as much as 75% for insufficient levels ([25-OH-D] <50nmol/L) (46-48).

The first conditions to be associated with vitamin D deficiency were related to disrupted mineral homeostasis, and consequential skeletal disorders such as osteoporosis and osteomalacia in adults, and rickets in children (8, 49). Hypovitaminosis D has subsequently been associated with a large range of diseases, including autoimmune diseases such as multiple sclerosis, cardiovascular disease, dementia, and inflammatory diseases such as periodontal disease (50-53). There is growing evidence that supplementation with vitamin D, or sufficient UVB exposure can have a prophylactic effect on these conditions (54).

1.2.5 Vitamin D and periodontal disease

As with a number of other inflammatory diseases, low circulatory 25-OH-D concentration has been associated with periodontal disease, which is a chronic inflammation of the periodontium (tissues supporting the dentition), the inflammation leads to destruction of the these tissues (55, 56). Currently there is no clear consensus on the association in the literature which can largely be attributed to inter-cohort variation, and a lack of standardisation across studies (57). Mechanistically, the association may be multifaceted in cause, with vitamin D known to regulate bone homeostasis, via plasma calcium concentration, and VDR mediated bone resorption (58, 59). Furthermore, vitamin D has been demonstrated to attenuate inflammatory responses via the vitamin D receptor, for example by inhibiting lipopolysaccharide (LPS) induced activation of monocytes and macrophages, and subsequent cytokine release (60). Cathelicidin (LL-37) is an important antimicrobial component of the innate immune system, inducing chemotaxis, increased vascular permeability, and is bactericidal by oligomerising in microbial membranes and disrupting their integrity (61). The expression of LL-37 is regulated by VDR, and is therefore regulated by the concentration of 1,25-$(OH)_2$-D (62). Limited data suggests supplementation of 1,25-$(OH)_2$-D in a periodontal disease mouse model reduces inflammation by upregulating LL-37 expression (63).

In vitro studies have demonstrated vitamin D metabolites can be protective against inflammation in periodontal ligament cells and gingival fibroblasts by modulating cytokine concentrations (64, 65). Limited data also suggests 1,25-$(OH)_2$-D may inhibit growth and

virulence of *P. gingivalis*, which is a keystone pathobiont species, strongly associated with periodontal disease, and reduce the severity of inflammation by inhibiting nuclear factor kappa B (NF-κB) function (66). 1,25-$(OH)_2$-D reduces NF-κB activity by increasing expression of I kappa B alpha (IκB-α), which in turn reduces translocation of NF-κB to the nucleus and therefore attenuates its function (67).

Clinical supplementation studies in a cohorts of periodontal disease patients have demonstrated modest improvements to clinically quantifiable parameters of periodontal disease severity, and indeed improved health of the periodontium has been observed in patients on maintenance therapy (68-70).

1.3 Vitamin B_{12}

1.3.1 Metabolism and physiology

Vitamin B_{12} or cobalamins, denotes a group of water-soluble organometallic compounds which are synthesised *de novo* by a small group of prokaryotic and archaeal species in a hydroxylated form (71, 72). Humans must source vitamin B_{12} from foods of animal origin as no reliable plant source. Structurally, vitamin B_{12} consists of two invariable moieties, a corrin ring, and nucleotide loop, and interchangeable R-group (Figure 1-2) (73). Physiologically active R-groups in humans are adenosyl- or methyl-cobalamin,

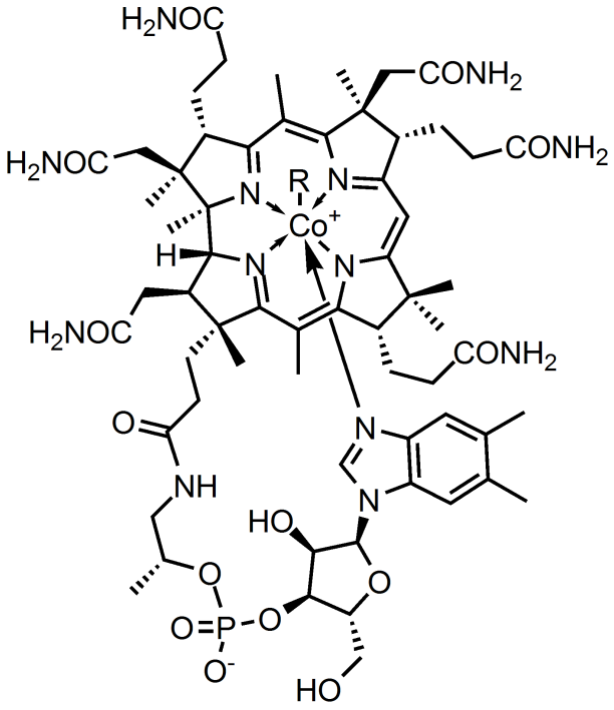

Figure 1-2 Chemical structure of vitamin B_{12} (cobalamin)
The R group is interchangeable. Microbially synthesised as OH-cobalamin, pharmaceutically converted to CN-cobalamin, physiologically active in humans as either Ado-Cobalamin or methyl-cobalamin.

though cyano-cobalamin is a commonly produced pharmaceutical analogue which is stable and easily processed (74).

Adenosylcobalamin (5'-deoxyadeadenosylcobalamin) is a cofactor for the mitochondrial protein methylmalonyl-CoA mutase, which catalyses the production of succinyl CoA from methylmalonyl CoA, an intermediary step in succinate biosynthesis (75). The cytoplasmic methionine synthase requires methylcobalamin as a cofactor, in the methyl-tetrahydrofolate (methyl-THF) form of folate dependent synthesis of methionine (76). The two substrates for these enzymes, methylmalonic acid (MMA) and homocysteine (Hcy) are often utilised as functional biomarkers of vitamin B_{12} status (77).

The aforementioned necessity of vitamin B_{12} for two key metabolic processes renders vitamin B_{12} essential for a number of fundamental cellular functions. As noted earlier, synthesis of the essential amino acid, methionine is vitamin B_{12} dependent, therefore protein synthesis and methylation are affected by hypovitaminosis B_{12}. Secondly, donation of the methyl group from methyl-THF, allows THF to be converted to methylene-THF which is an important intermediary in thymidylate synthesis, and ultimately DNA replication (76).

1.3.2 Assimilation

Vitamin B_{12} (cobalamin) is present in the diet in very low concentrations, although essential for life, its requirement is also low. A complex multi-step pathway (Figure 1-3) has evolved to assimilate a high proportion of dietary vitamin B_{12}. Three homologous vitamin B_{12} binding proteins facilitate three distinct steps in uptake and transport. Firstly, haptocorrin (Hc) (please refer to 1.3.3), a salivary protein which is also expressed by the gastric mucosa is highly glycosylated and is thought to protect vitamin B_{12} from hydrolysis in the acidic environment of the stomach upon dissolution from food (78). The complex passes into the duodenum where the luminal pH gradient increases reducing the binding affinity of haptocorrin for the vitamin (79). Pancreatic proteases trypsin and pepsin cleave the binding protein releasing cobalamin (80). Hypovitaminosis B_{12} is observed in ~30% of patients suffering pancreatic insufficiency (i.e. a failure to secrete the correct cocktail of proteases and buffering compounds), due to haptocorrin-cobalamin complexes remaining intact, which are not recognised by receptors expressed in the gastrointestinal tract (80-83). Liberated cobalamin in the proximal small intestine is bound by a second binding protein, termed intrinsic factor (IF) which is secreted by parietal cells located in the gastric mucosa, but fails to bind cobalamin at low pH, and therefore binding occurs in the duodenum where the pH is neutralised (80, 84). IF-cobalamin complexes are

absorbed across the brush boarder membrane (BBM) by interacting with the multiligand receptor cubilin (CUB) and amnionless (AMN), these two proteins form the cubam complex which facilitates endocytosis, and are expressed in the terminal ileum (85, 86).

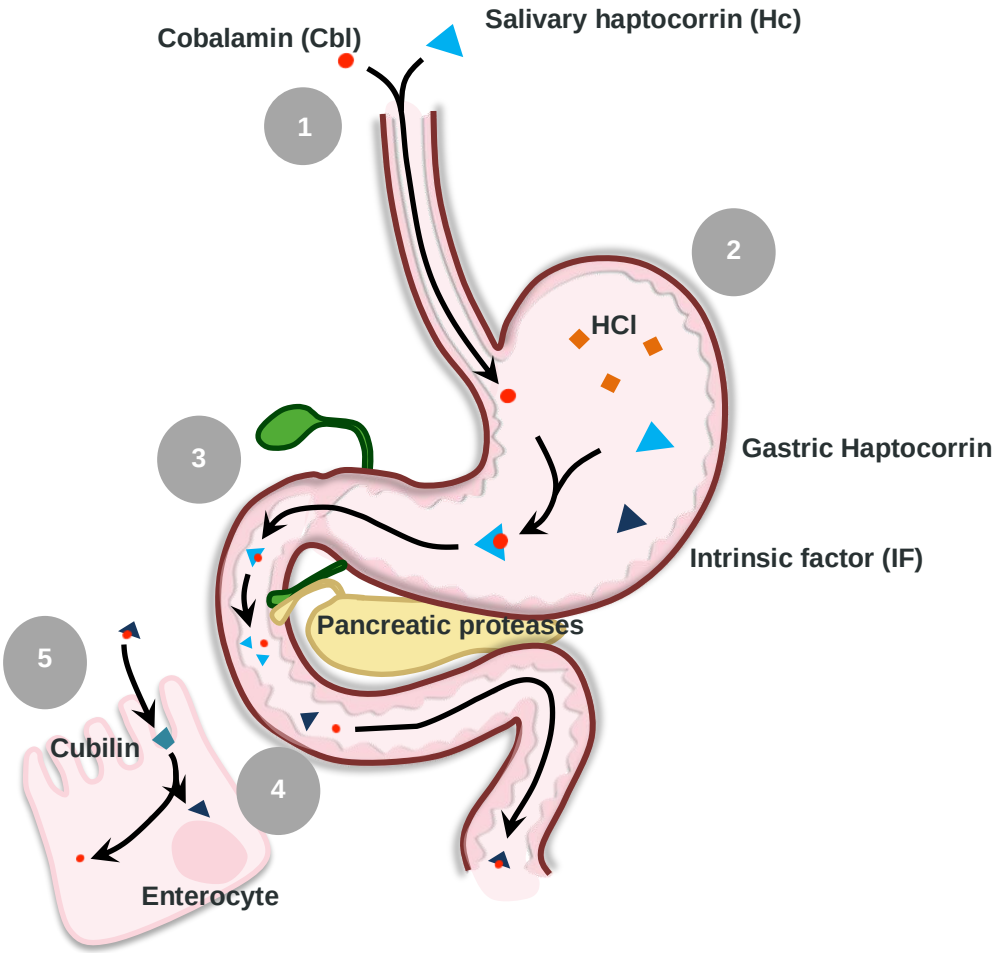

Figure 1-3 Canonical cobalamin assimilation pathway
1 - Cobalamin released from food proteins binds haptocorrin with a strong affinity at low pH. 2 - Extensive glycosylation of haptocorrin confers protection from hydrolysis in the gastric acidic milieu. 3 - Cobalamin is released in the duodenum with the increased pH and proteolytic degradation of haptocorrin. 4 - Cobalamin binds intrinsic factor, a homologous binding protein with greater specificity for physiologically active analogues, expressed by gastric parietal cells. 5 - IF-Cbl complex travels to the distal ileum where endocytosis is facilitated by cubilin.

Within the enterocyte lysosome intrinsic factor is cleaved (87). Free cobalamin is transported into the circulation via the ABC transporter, multi-drug resistant protein 1 (MRP1), expressed on the basolateral membrane (88). In plasma, cobalamin is bound by the third binding protein, transcobalamin-II (TCN-II) (89). Only the circulatory fraction of cobalamin bound to TCN-II is accessible, and is taken up by cells by the TCN-II receptor (TCN-II R) (90).

1.3.3 Haptocorrin

Haptocorrin (Hc), also referred to as transcobalamin-I (TCN-I) or R-binder is a 48KDa glycoprotein present in numerous exocrine secretions including saliva, tears, and milk

and is also expressed by granulocytes (91, 92). Haptocorrin is distinct from the other two transport proteins (intrinsic factor and transcobalamin-2) in that it is able to bind physiologically inactive analogues of cobalamin as well as those required by humans (93, 94). Haptocorrin is also present in serum and binds the majority of circulatory vitamin B_{12}; however, this fraction is only available to hepatocytes via the asialoglycoprotein receptor (95, 96). There is limited data in the literature which implies the potential antimicrobial function of haptocorrin, which is suggested to be a result of vitamin B_{12} sequestration, for many bacteria cobalamins are exogenously sourced micronutrient and therefore a deficiency can be growth limiting (97-99).

1.3.4 Epidemiology and manifestations of hypovitaminosis B_{12}

The predominant aetiology of vitamin B_{12} deficiency varies across the globe. For populations following vegetarian and especially vegan diets which have low cobalamin concentrations, dietary insufficiency is typically the most prevalent source of deficiency (100). In communities consuming diets rich in vitamin B_{12}, malabsorption is generally the most frequently attributed cause. Malabsorption of vitamin B_{12} can occur for a number of reasons including, pancreatic insufficiency, a lack of intrinsic factor commonly as a result of parietal cell atrophy, or inflammatory disorders of the terminal ileum (101-103). Vitamin B_{12} deficiency in the United Kingdom is particularly prevalent in the elderly with 5% 64-74-year olds being deficient, rising to 10% in the over 75s. Deficiency prevalence is even higher in the vegan population, to almost 50% (104, 105).

Due to the fundamental importance of vitamin B_{12} to metabolic and cellular processes, deficiency can manifest in a number of ways. Typically, megaloblastic anaemia is an early indicator of deficiency which occurs due to disruption of DNA synthesis, desynchrony between nuclear and cytoplasmic maturation occurs and disrupts mitosis (106). If left untreated, vitamin B_{12} can cause other issues and can have serious neurological implications such as motor disturbances, memory loss and even psychosis, attributed in part to demyelination (106).

1.3.5 Vitamin B_{12} and periodontal disease

The literature supporting an association between vitamin B_{12} status and periodontal disease is small, with one study demonstrating an inverse association between markers of vitamin status (total-B_{12}) and clinical indicators of periodontal disease progression such as depth of the gingival pocket (PD), and attachment loss of the gingival tissue (CAL) (107).

1.4 Vitamin A

1.4.1 Metabolism and physiology

Vitamin A denotes a group of lipid-soluble compounds described as provitamin A, which comprises carotenoids of plant origin: α-carotene, β-carotene, and β-cryptoxanthin, which are highly abundant in carrots and collard greens (108, 109). Preformed vitamin A refers to retinyl esters and retinol (Figure 1-4 (i)) which are sourced from animal dietary components such as cod liver and eggs (110). The functions of retinoids can broadly be grouped into the production of 11-*cis*-retinal (Figure 1-4 (ii)), which is required for vision, and the production of retinoic acid (RA) (Figure 1-4 (iii)), which is required for modulating gene expression and development.

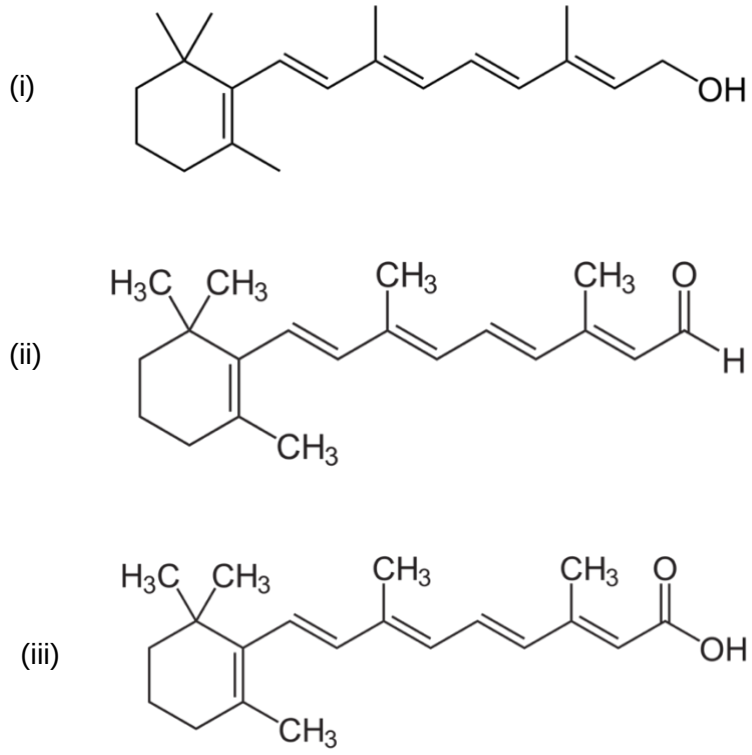

Figure 1-4 Chemical structure of vitamin A metabolites
(i) retinol, (ii) retinal (iii) retinoic acid,

The importance of vitamin A for vision has been well delineated. A chromophore vitamin A derivative, 11-*cis*-retinal, binds rhodopsin and cone opsins in photoreceptors. Photons induce the isomerisation of this derivative from *cis*- to *trans*- coordination which is critical for the transduction of sight (111). It is becoming increasingly apparent that vitamin A is essential for a number of other functions such as regulation of immunity, particularly at mucosal surfaces by modulating receptor expression such Toll-like receptor 2 and sIgA concentration (112), and cellular differentiation and morphogenesis (113). Vitamin A in

the forms all-*trans*-retinoic acid, and 9-*cis*-retinoic acid, are the ligands for the nuclear receptors; retinoic acid receptor (RAR), and retinoid X receptor (RXR), which bind to the widely-occurring retinoic acid response elements (RAREs) and subsequently regulate gene expression (114, 115).

1.4.2 Assimilation

Vitamin A and the other lipid soluble vitamins are considered to emulsify into mixed micelles as they dissolute from the food matrix and separate into the lipid fraction of the meal. Studies have shown that vitamin A is incorporated into chylomicron and micelles, with very little processing in the stomach, excluding hydrolysis of retinyl-esters (116). Previous research undertaken by Hollendar *et al.* in the 1970s presented data suggesting carrier-protein mediated passive diffusion is responsible for gastrointestinal uptake of vitamin A analogues; however, these studies undertaken on *ex vivo* rat intestines, were using pharmaceutical concentrations (117, 118). More recent work has focused on physiological concentrations of vitamin A, and transit across enterocytes via receptor mediated pathways (119). The steroid receptors described in the assimilation of vitamin D (SR-B1, CD36, and NCP1L1) are also implicated in receptor mediated uptake of vitamin A from the gastrointestinal lumen (119). Animal models have been a useful tool in deciphering the site of gastrointestinal absorption. Uptake is detectible in the small intestine and is minimally so in the caecum and colon (120). Vitamin A circulates predominantly bound to retinol-binding protein RBP (121). Cellular uptake of the RBP-retinol complex is facilitated by “stimulated by retinoic acid-6” (STRA6), a widely expressed, specific transmembrane transporter (122, 123).

1.4.3 Retinol-binding protein

Retinol-binding protein (RBP/ RBP4) is a 21kDa plasma protein, expressed predominantly by hepatocytes, and to a lesser extent in more distal tissues such as renal parenchyma and adipose (124, 125). RBP circulates bound to another hepatically expressed 55kDa protein; transthyretin, which increases the molecular weight of the complex and therefore reduces loss during glomerular filtration (126). Recent developments suggest RBP may function as an adipokine and be an indicator of insulin resistance in type 2 diabetes though this remains contentious in the literature (127).

1.4.4 Epidemiology and manifestations of hypovitaminosis A

Hypovitaminosis A remains a major global health problem with an estimated prevalence of 130 million children, and 7 million pregnant women in lower-income countries, and is predominantly attributed to dietary insufficiency, and to a lesser extent malabsorption

(128). Extremes of vitamin A status hypo-/hyper-vitaminoses A, has been attributed to increased mortality in the elderly (129). The supportive function of vitamin A to immunity means that deficiency is associated to a high rate of child mortality from infectious diseases, particularly diarrheal attributed mortality (130).

Vitamin A deficiency can manifest is a variety of ways, most acutely as night-blindness, which is a reduced ability to adapt to reduced light intensity, this is the most common symptom of xerophthalmia (131). Xerophthalmia is a collection of ocular conditions associated with hypovitaminosis A, including dryness, and inflammation (132). Vitamin A deficiency is associated with infections and reduced mucosal barrier function (133, 134).

1.4.5 Vitamin A and periodontal disease

There is increasing evidence that oxidative stress (OS) can have negative health implications for the oral cavity, including periodontal tissues (135, 136). Sources of OS in the oral cavity are numerous and include, food, inflammation and smoking (136). Nutritional studies have indicated that, dietary or supplemented antioxidants, such as pro-vitamin A carotenoids, α-tocopherol (vitamin E), and vitamin C can increase the rate of healing in periodontal tissue when assessed by pocket depth in non-smoking periodontal disease patients (137).

1.5 Saliva and vitamin-binding proteins

Whole mouth saliva (WMS) is the complex product of three pairs of major salivary glands, numerous minor glands, as well as contaminants from the gingival crevicular fluid, the oral epithelia, microbiome and consumed items (138). Typical resting whole mouth saliva secretion is 0.3-0.4mL/minute although there is great range (139). Saliva contributes to the efficient functioning of the oral cavity, and upper digestive tract. Members of the complex salivary proteome include the aforementioned three vitamin-binding proteins; vitamin D-binding protein (DBP), haptocorrin (Hc), and retinol-binding protein (RBP) which have been detected in a number of exocrine secretions, including saliva (91, 140, 141).

1.5.1 Anatomy of the major salivary glands

Whole mouth saliva is the product, in part, of the major salivary glands which are bilaterally paired exocrine glands consisting of the parotid, sublingual, and submandibular glands (1). Ducts channel the saliva into the oral cavity onto the oral mucosa (Figure 1-5).

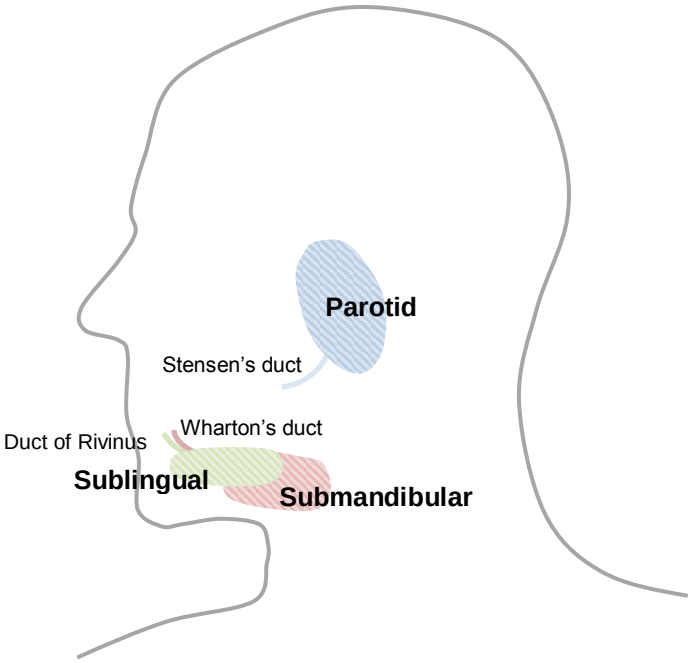

Figure 1-5 Anatomy of the left side major salivary glands and corresponding major ducts.

Salivary gland parenchyma is composed of two distinct epithelial tissues. Firstly, the acinar epithelium, responsible for secreting salivary fluid, as well as the majority of the salivary proteome, and secondly, the ductal epithelium which modify salivary ionic content as well as contributing some proteins as saliva is channelled into the oral cavity (142). Parenchymal tissue is arranged into lobes separated by connective tissue which is densely populated by nerve fibres, vasculature, and myoepithelial cells (143).

The microstructure differs between the major glands and consequently influence the composition properties of their secretions (144). One key variance between the glands is the composition of acinar cell types within the parenchyma, defined by the secretory proteins they synthesis. Secretions from the various glands are typically divided into mucinous or non-mucinous (Figure 1-6). Histological staining of the major glands with haematoxylin and eosin demonstrates the parotid gland acinar cells are predominantly serous (non-mucinous) and sublingual being largely mucinous, the submandibular glands demonstrate mixed populations (1).

The parotid and submandibular glands contain intercalated, striated, and excretory ducts, the sublingual gland however, lacks striated ducts. Striated ductal cells have the machinery required for ion resorption which is responsible for the hypotonicity of these secretions (145).

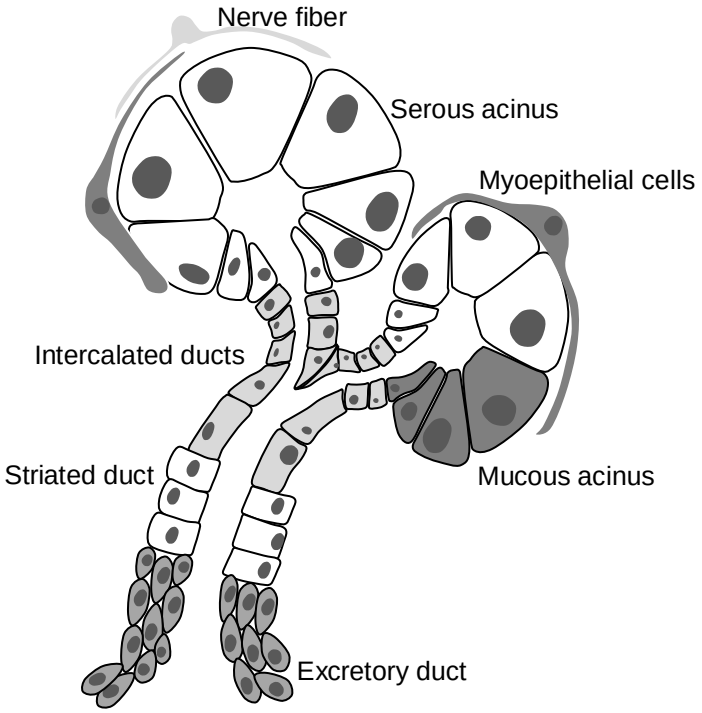

Figure 1-6 Architecture of a generalised salivary gland

1.5.2 Saliva secretion

Saliva secretion by the major glands is stimulated by chemoreceptors and other taste receptors, located within taste buds, primarily distributed in the dorsal epithelium of the tongue, and by mechanoreceptors within the periodontal ligaments and mucosa (146, 147). The salivary reflex has been well delineated, in brief, afferent signals from the aforementioned receptors in the oral cavity are transmitted to the trigeminal nucleus, and nucleus solitary tract, which in turn transmit signals to the salivary nuclei. Efferent signals are subsequently transmitted via the glossopharyngeal, and lingual nerve, to the parotid, and sublingual/ submandibular glands respectively (1). Innervation of the salivary glands is controlled by both sympathetic and parasympathetic branches of the autonomic

nervous system. Parasympathetic innervation of the glands is mediated by acetylcholine activation of muscarinic receptors leading to ion and water secretion. Sympathetic release of noradrenaline activates β1-adrenergic receptors, stimulating protein secretion (148). (Acinar secretion of saliva is summarised in Figure 1-7). Salivary flow rate and composition can vary under circadian control and the type of stimulant; therefore, it can be beneficial to present salivary proteins as an output.

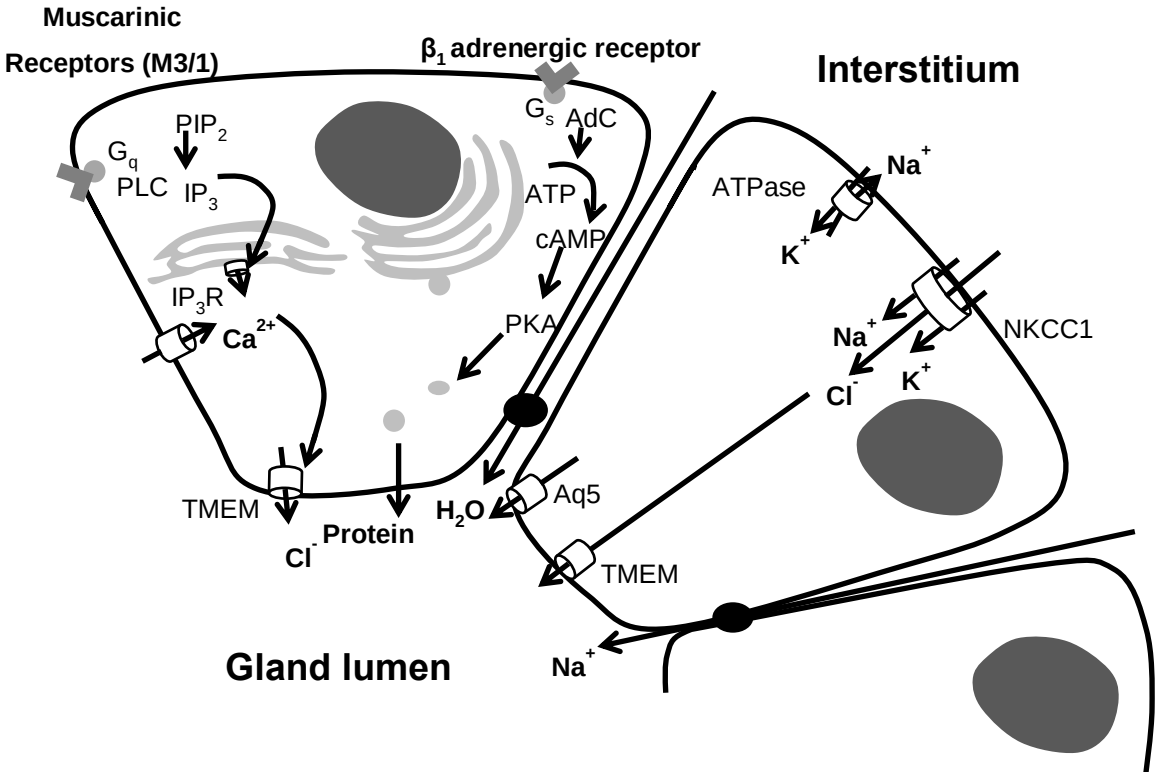

Figure 1-7 Simplified cellular processes of acinar saliva secretion

Acinar Ion and water secretion

Fluid secretion is driven by parasympathetic stimulation of muscarinic receptors. Polarisation of acinar cells features distinct localisation of different ion on water receptors to facilitate the direction of secretion into the glandular lumen. The ATP dependent sodium pump in the basolateral membrane of acinar cells maintains a low intracellular sodium concentration, inducing the movement of chloride and sodium ions through the cell into the acinus lumen which creates an osmotic gradient. This induces water to follow either intercellularly or intracellularly via aquaporin 5 (Ag5), thus creating the fluid component of saliva.

Acinar protein secretion

Protein secretion is driven by sympathetic stimulation of β1-adrenergic receptor activation which in-turn increases the cytosolic cAMP concentration, and activation of protein kinase A (PKA), subsequently exocytosis is induced.(1)

AdC	Adenylate cyclase	**IP_3**	Inositol triphosphate
Aq5	Aquaporin 5	**IP_3R**	Inositol triphosphate receptor
ATP	Adenosine triphosphate	**K^+**	Potassium
Ca^{2+}	Calcium ion	**Na^+**	Sodium ion
cAMP	Cyclic AMP	**NKCC1**	Sodium, potassium, chloride co-transporter
Cl^-	Chloride ion	**PIP_2**	Phosphatidyl inositol bisphosphate
G_q	G protein q	**PKA**	Protein kinase A
G_s	G protein s	**PLC**	Phospholipase C
H_2O	Water	**TMEM**	Calcium activated chloride channel

1.5.3 Sources of the whole mouth salivary proteome

The majority of salivary proteins are expressed by the salivary glands, though there is great inter-glandular variation in which proteins are synthesised (144). As illustrated by Figure 1-7, proteins expressed by acinar cells are packaged densely into secretory granules with the aid of calcium ions to balance charge, proteins are released by exocytosis upon autonomic innervation of the glands (see number 3, Figure 1-8) (149, 150). Saliva contains proteins from blood, interstitial, and plasma cell proteins (such as sIgA) which can enter the end piece lumen either paracellularly (see number 1, Figure 1-8), or transcellularly (see number 2, Figure 1-8) (151, 152). Whole mouth saliva further contains proteins from shed oral epithelial cells, the transudate gingival crevicular fluid, and from the oral microbiota.

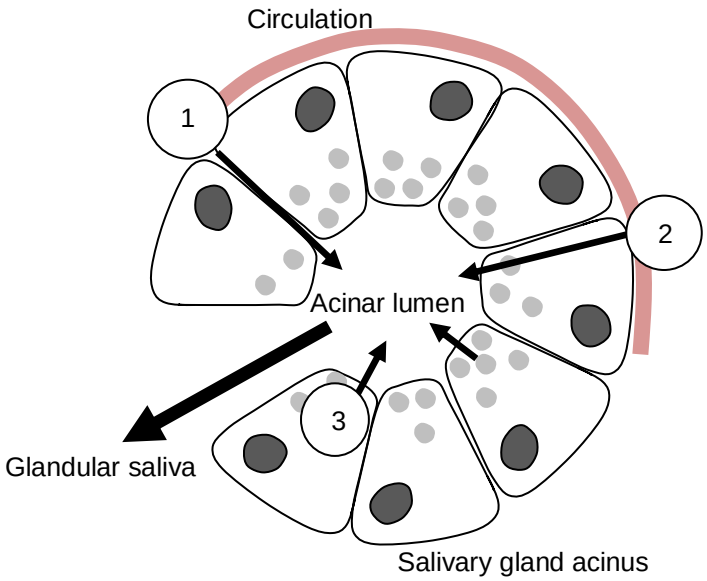

Figure 1-8 Glandular sources of salivary proteins.
1 – Blood proteins. 2 – IgA from plasma cells. 3 – Exocytosis of acinar storage vesicles containing proteins.

1.5.4 Saliva composition and functions

Saliva is biofluid composed of ions and a complex proteome of ~2000 proteins (152). The diversity of salivary constituents facilitates the numerous functions of saliva in maintaining the roles of the oral cavity. Although saliva is ~99% water, its constituents render it a non-Newtonian fluid (i.e. it demonstrates reduced viscosity with increased shear forces). Shear forces are common in the oral cavity, for example during chewing, and this property facilitates lubrication for the mechanical functions in the mouth (153, 154). The non-Newtonian attributes of saliva are in part attributable to the large molecular weight glycoproteins; mucins (155). Mucins also add to the adhesive quality of saliva (the

extensional rheological property termed spinnbarkeit), this facilitates the ability of saliva to coat the oral surfaces (156). By coating the structures of the mouth, saliva is able to facilitate many other functions. Saliva is a medium which enables the dissolution of tastants from the food matrix and detection by receptors expressed in the oral mucosa (157, 158). Saliva has further been shown to be essential for detection of the basic taste modalities (i.e. sweet, sour, bitter, umami, and salt) as well as more complex perceptions such as astringency (159). The physical properties of saliva are also essential for effective swallowing of food, in addition to its lubricating ability, the adhesive nature of saliva allows the aggregation of the food particles produced during mastication, to form a bolus prior to swallowing (160).

Further to the gustatory functions of saliva, it is critical for homeostasis of the oral cavity. Adsorption of salivary proteins onto both the oral hard and soft tissues offers a protective layer termed the pellicle (mucosal and enamel) resisting abrasion, erosion and facilitating microbial adhesion (161-163). Numerous antimicrobial components are present in saliva which demonstrate anti-bacteria (alpha-defensins), anti-viral (cystatins), and anti-fungal (cathelicidin) properties (164). Components such the growth factors; EGF and VEGF are also present and aid repair of oral tissues (165).

1.6 Periodontal disease and the pathobiont *Porphyromonas gingivalis*

As noted in sections 1.2.5, 1.3.5, 1.4.5, some literature demonstrates associations between systemic micronutrient status and periodontal disease. Periodontal disease is a collection of inflammatory disorders affecting the supportive tissues of the dentition. A milder form of gum inflammation is gingivitis which involves inflammation of the soft tissues around the teeth (gingiva) in response to the adjacent biofilm (plaque) which develops in the gingival sulcus. Gingivitis can usually be rectified with more thorough oral hygiene. If left untreated, gingivitis can develop into periodontitis (166). Periodontitis is a destructive inflammatory condition of both hard and soft tissues supporting the teeth, which can lead to attachment loss of the periodontal ligament to the dentition and resorption of the alveolar bone, eventually resulting in tooth-loss. The classification system of periodontal disease was recently redefined (2017) and now considers the aetiology when classifying the disease (167, 168). The aetiology of periodontal inflammation is considered to be a disruption of host homeostasis by dysbiosis in the subgingival plaque, this is followed by an overzealous immune response leading to tissue destruction (169).

1.6.1 Epidemiology of periodontal disease

Periodontal disease is one of the two most prolific inflammatory diseases worldwide and a significant contributor to chronic disease globally (170). The global prevalence of periodontal disease is ~10-15% in adults (171). Susceptibility to periodontal disease is known to be related to a number of genetic factors as well as environmental such as nutrition, oral hygiene, smoking status, and systemic diseases the most notable of which being diabetes (172, 173).

1.6.2 Commensal oral microbiota

Colonisation of the oral cavity by complex communities of microbes including, bacteria, viruses, fungi, and archaea is a natural feature of healthy oral homeostasis (174). Typically, the commensal species support the healthy environment of the oral cavity by outcompeting, or even inhibiting proliferation of more pathogenic species (175). Interestingly the oral microbiota has recently been demonstrated to have implications for systemic hypertension. Nitrate in saliva is a substrate for certain bacteria and is reduced to nitrite, which in turn is a precursor of anti-hypertensive nitric oxide which regulates endothelial function in the vasculature (176, 177).

Adsorbed salivary proteins on oral surfaces offer a natural barrier to limit direct contact between microbes and the oral mucosa, and frequent swallowing limits microbial biomass accumulation (178). Discrete environmental niches in the oral cavity leads to site specific microbial ecology (179). Niches within the oral cavity include the gingival sulcus, the enamel surface, the tongue, the hard and soft pallets, and oral prostheses (180).

1.6.3 Microbial dysbiosis

During the transition into a periodontal disease state, the subgingival plaque composition shifts from a diverse community of predominantly Gram-positive species to a more restricted community of Gram-negative anaerobes (181). Analysis of plaque has indicated close association of microbial species including *Porphyromonas gingivalis, Tannerella forsythia,* and *Treponema denticola* and an enrichment of their virulence factors at sites of inflammation (182, 183). This cohort of species is termed the “red complex” (184). Of the red complex, *Porphyromonas gingivalis* is considered the “keystone species”, although present in low amounts, even in a disease state, its virulence factors can direct the dysbiosis of the subgingival microbiota which is a key driver of periodontal inflammation (185).

1.6.4 *Porphyromonas gingivalis* and its cysteine proteases

Porphyromonas gingivalis (*P. gingivalis*) is Gram-negative anaerobic bacterial species with a strong association to periodontal disease (186). Meta-analysis has indicated *P. gingivalis* is present in 78% of periodontal disease patients, compared to just 34% of periodontally healthy controls (187). *P. gingivalis* possesses an arsenal of key virulence factors which allow it to act as a keystone species and modulate not only plaque composition, but also host immune response (188). One of the key virulence factors of *P. gingivalis* is a group of promiscuous cysteine proteases termed “gingipains” (189). Gingipains are both secreted, or membrane associated, and consist of two arginine-specific proteases RpgA/B, and a lysine-specific protease Kgp (189). Expression of gingipains is regulated in response to environmental factors, such as nutrient availability, for example haemin concentration (190, 191). Significant literature demonstrates the contribution of gingipains to subversion of immunity, tissue infiltration, and nutrient requisition. Arg-X gingipains in particular, have been shown to first stimulate a localised immune response, therefore providing essential nutrients, at high enough concentrations gingipains cleave chemotactic compliment components C3 and C5, thus reducing neutrophil recruitment (192, 193). Gingipains are essential for a number of nutrient sourcing processes, one important process is in liberating haemin from erythrocytes (194). Searching for novel virulence factors which support *P. gingivalis* fitness continues to be a focus in the literature with the generation of transposon mutant libraries, which can be a high throughput method for highlighting essential genes (195).

1.6.5 *Porphyromonas gingivalis* and the cobalamin biosynthesis pathway

As noted in chapter 1.3, the cobalamin biosynthesis pathway is a complex ~30 enzyme-dependent process conserved in a small number of microbial species. The pathway has been delineated and demonstrates at least two distinct routes either aerobic or anaerobic (196). Extracts of various *P. gingivalis* strains has demonstrated a lack of activity for two essential enzymes in the anaerobic pathway, and this is supported by genomic analysis. It is proposed the remainder of the pathway is conserved as a scavenging pathway for conversion of inactive analogues (197). There is no literature which assesses the influence of haptocorrin on *P. gingivalis* growth.

1.7 Thesis aims and objectives

Although researched in detail in the circulation, the functions of vitamin D-binding protein, haptocorrin, and retinol-binding protein, in the context of saliva, have been minimally described in the literature. The overall aim of this thesis is to determine if vitamin-binding proteins present in saliva contribute to dietary vitamin assimilation, and to understand the influence they have on microbial growth. To achieve this, the following objectives will determine how well salivary and serum concentrations correlate in healthy controls, whether vitamin-binding proteins change during inflammation, and if they inhibit growth of bacteria.

1.7.1 Objectives

The key objectives of this thesis are:

1. **To understand the relationship between salivary and serum vitamin-binding protein concentrations with systemic vitamin status**
   Saliva has great potential to be a non-invasive source of biomarkers which can be readily collected. Understanding the relationship between salivary vitamin-binding proteins and systemic markers of vitamin status may offer salivary marker of vitamin status.
2. **To determine the contribution of different salivary glands to whole mouth salivary concentrations, and the influence of stimulation on their concentrations**
   The concentrations of vitamin-binding proteins between resting and stimulated salivary flow may be suggestive of their function within the oral cavity, increased output with stimulation may be important for aiding dietary vitamin absorption.
3. **To understand the influence of periodontal disease on salivary vitamin-binding protein concentration, and systemic vitamin status**
   Periodontal disease is associated with a number of micronutrient deficiencies, is this a consequence of disrupted salivary vitamin-binding protein function?
4. **To understand the interactions between haptocorrin, and the keystone perio-pathobiont: *Porphyromonas gingivalis***
   Vitamin B_{12} is a nutritional requirement for a number of microbial species including a key aetiological species for periodontal disease. *P. gingivalis* lacks the complete anaerobic cobalamin biosynthesis pathway and therefore must source it exogenously. Haptocorrin, may therefore exert an innate immune function by withholding an essential micronutrient.

Chapter 2 Common materials and methods

This section describes the materials and methods common across multiple results chapters. Chapter specific methods will be covered within each chapter.

2.1 Protein biochemistry methods

2.1.1 Quantification of total protein by bicinchoninic acid assay

Total protein concentration of all sample types was determined by bicinchoninic acid assay (BCA) (ThermoScientific, Waltham, MA, U.S.A). Equal volume of samples was added to a 96-well plate in duplicate. Albumin standard (2mg/mL) was serially diluted to generate an appropriate series. Fresh working reagent was then prepared and added to all wells. The plate was sealed and incubated for thirty minutes at 37°C, absorbance was measured immediately after at 540nm using iEMS 96 well microplate reader (ThermoFisher, Waltham, MA, U.S.A.). Absorbance of the albumin titration was used to generate a standard curve from which the protein content of samples was generated.

2.1.2 Western blot

Samples were prepared in NuPAGE lithium dodecyl sulphate sample buffer (ThermoFisher, Waltham, MA, U.S.A.) and reduced with 1,4-Dithiothreitol (DTT) prior to heating to 100°C for two minutes. The protein profile of saliva and molecular weight standard, SeeBlue Plus2 (ThermoFisher, Waltham, MA, U.S.A.) was separated using XCell Sure Lock Mini-Cell electrophoresis system on NuPAGE 4-12% Bis-Tris gels (ThermoFisher, Waltham, MA, U.S.A.), for thirty minutes at a constant of 200v.

Proteins were transferred onto supported nitrocellulose membrane (Bio Rad, Hercules, CA, U.S.A.) over an hour at a constant of 30v.

2.1.3 Immunoprobing

Blots were blocked with 5% w/v skimmed milk powder (Sigma-Aldrich, St. Louis, MI, U.S.A) suspended in tris buffered saline with 0.1% $tween_{20}$ (TBST) for one hour at room temperature with gentle agitation. Blots were incubated for one hour at room temperature with the following antibodies diluted in blocking agent.

Table 2-1 Primary antibodies used for immunoblotting

Target immunogen	Clonality	Host	Working concentration	Manufacturer
Haptocorrin	Monoclonal	Mouse	0.25µg/mL	Novus Biologicals, Abingdon, U.K.
Vitamin D-binding protein	Polyclonal	Rabbit	0.30µg/mL	Abcam, Cambridge, U.K.
Retinol-binding protein	Monoclonal	Rabbit	0.23µg/mL	Abcam, Cambridge, U.K.

Blots were then washed three times for five minutes at room temperature in TBST. Secondary antibody incubation was undertaken with the following, also in blocking agent.

Table 2-2 Secondary antibodies used for immunoblotting

Target immunogen	Conjugate	Host	Working concentration	Manufacturer
Mouse Ig	HRP	Goat	0.50µg/mL	Dako, Santa Clara, CA, U.S.A.
Rabbit Ig	HRP	Swine	0.65µg/mL	Dako, Santa Clara, CA, U.S.A.

Blots were again washed three times for five minutes at room temperature with TBST. Bands were visualised by chemiluminescence using ChemiDoc XRS+ (Bio-Rad Laboratories, Hercules, CA, U.S.A.) with enhanced chemiluminescence substrate (ECL) (Bio-Rad Laboratories, Hercules, CA, U.S.A.) in order to detect horseradish peroxidase (HRP) activity which is the reporter system conjugated to the secondary antibodies. Equal volumes of ECL system were mixed and applied to the membrane for 20 seconds, excess substrate was removed. Sequential imaging tool place to produce clear images prior to over exposure. Image Lab software (Version 4.0 build 16) (Bio-Rad Laboratories, Hercules, CA, U.S.A.) was used for analysis.

2.1.4 Quantification of haptocorrin by ELISA

Haptocorrin was quantified by sandwich enzyme-linked immunosorbent assay (ELISA). All reagents were provided in kit form (Cusabio biotech Co Ltd, Houston, TX, U.S.A.). Neat saliva and serum samples were assayed in duplicate on a 96-well plate precoated with monoclonal anti-haptocorrin antibody along with a titration of standard. Wells were aspirated and washed thoroughly five times with wash buffer. Another monoclonal anti-haptocorrin biotinylated antibody was added to all wells and incubated at room temperature for thirty minutes. Wells were aspirated and washed thoroughly five times with wash buffer. Avidin-HRP conjugate was then added to each well and incubated for a further thirty minutes. Wells were aspirated and washed thoroughly five times with wash buffer. Two substrate solutions (stabilised hydrogen peroxide followed by tetramethylbenzidine) were added in sequence and incubated for ten minutes at 37°C while restricted from light. Stop solution (6N sulphuric acid) was added and optical density values were measured at 450nm with an iEMS 96 well microplate reader (ThermoFisher, Waltham, MA, U.S.A.). The mean of duplicate standard and sample values were calculated, and the blank was subtracted from all values. Optical density

readings were plotted against corresponding concentrations on a scatterplot, a line of best fit was applied. The trend line equation was rearranged in order to calculate individual sample concentrations.

2.1.5 Quantification of vitamin D-binding protein by ELISA

Vitamin D-binding protein was quantified by sandwich enzyme-linked immunosorbent assay (ELISA). All reagents were provided in kit form (R&D Systems, Inc. Minneapolis, MN, U.S.A.). Saliva and serum samples were diluted two-fold, and two thousand-fold respectively in calibrator diluent, and loaded in duplicate onto a 96-well plate precoated with a monoclonal anti-DBP antibody, along with the provided standards, and incubated for two hours at room temperature with horizontal orbital rotation of 450rpm using iEMS 96 well microplate reader (ThermoFisher, Waltham, MA, U.S.A.). Wells were then aspirated and thoroughly washed four times with wash buffer. Anti-DBP monoclonal antibody HRP-conjugate was then added to each well and incubated for one hour, again with horizontal orbital rotation at room temperature. Wells were again aspirated and washed four times. Substrate solution (1:1 ratio of stabilised hydrogen peroxide and tetramethylbenzidine) was then added for thirty minutes and incubated at room temperature without agitation and excluded from light. Stop solution (2N sulphuric acid) was then added, optical density was determined with iEMS 96 well microplate reader (ThermoFisher, Waltham, MA, U.S.A.) at wavelength 450nm, readings were also taken at 540nm and subtracted from the former to adjust for optical imperfections on the plate. The mean of duplicate standard and sample values were calculated, and the blank was subtracted from all values. Optical density readings were plotted against corresponding concentrations on a scatterplot, a line of best fit was applied. The trend line equation was rearranged in order to calculate individual sample concentrations.

2.1.6 Quantification of retinol-binding protein by ELISA

Retinol binding protein was quantified by sandwich enzyme-linked immunosorbent assay (ELISA) all reagents were provided in kit form (Invitrogen, Frederick, MD, U.S.A). Saliva and serum samples were diluted two-fold and one hundred-fold respectively in sample diluent. Samples and standards were added to the precoated monoclonal anti-RBP antibody assay plate biotin-conjugated monoclonal anti-RBP antibody was then added and incubated for two hours at room temperature with orbital agitation (400rpm). Wells were aspirated and washed four times thoroughly with wish wash solution. Streptavidin-HRP conjugate was then added to all wells and the plate was incubated at room temperature with orbital agitation (400rpm) for one hour. Wells were then aspirated, and washing was repeated a further four times with wash buffer. Detector solution

(tetramethylbenzidine) was added to each well and incubated for thirty minutes at room temperature, excluded from light. Stop solution (1M phosphoric acid) was added to each well and optical density was measured at 450nm with an iEMS 96 well microplate reader (ThermoFisher, Waltham, MA, U.S.A.). The mean of duplicate standard and sample values were calculated, and the blank was subtracted from all values. Optical density readings were plotted against corresponding concentrations on a scatterplot, a line of best fit was applied. The trend line equation was rearranged in order to calculate individual sample concentrations.

2.2 Vitamin quantification

2.2.1 Determination of systemic Vitamin B_{12} and D status

Total-vitamin B_{12}, and 25-hydroxyvitamin D were quantified in serum samples as markers of systemic vitamin B_{12} and vitamin D status respectively. Quantification was undertaken by chemiluminescent microparticle immunoassay (CMIA) on an ARCHITECT i *system* (Abbott Diagnostics, Maidenhead, U.K.) at the Viapath Nutristasis Unit (St. Thomas' Hospital, London). This is an automated high-throughput system used clinically. Daily calibration and controls are undertaken on the machine with known concentrations of each analyte to generate a standard curve. 200µL of sample are applied to sample cups (Abbott Diagnostics, Maidenhead, U.K.). Paramagnetic microparticles coated with monoclonal antibodies for the two metabolites are added to samples by automation. Post incubation, analytes bound to the microparticles are precipitated by magnetism and excess sample is aspirated, the microparticles are washed with buffer and resuspended in sample buffer which contains acridinium-labelled monoclonal antibodies targeted to the analyte. The precipitation and was steps are repeated, before hydrogen peroxide is added to each sample cub which induces the production of chemiluminescence which is measured as relative light units (RLUs). RLUs has a direct relationship to the analyte tested.

2.2.2 Determination of systemic Vitamin A status

Retinoic acid was quantified in serum samples as markers of systemic vitamin A status. Quantification was undertaken by high-performance liquid chromatography (HPLC) at the Viapath Nutristasis Unit (St. Thomas' Hospital, London). The following set up was used: Dionex Ultimate 3000 pump and autosampler (ThermoFisher, Waltham, MA, U.S.A.) coupled with UV detector at 975nm (Jasco, great Dunmow, U.K.) BetaSil C18 reverse phase column (3µm 100x4.6mm) (ThermoFisher, Waltham, MA, U.S.A.).

2.3 Statistical analysis

All statistical analysis, and plot generation was undertaken using Prism 7 for Mac OS X Version 7.0a April 2, 2016 (GraphPad Software, Dan Diego, CA, U.S.A.). Specific statistical analyses used will be covered in each chapter.

Chapter 3 Salivary and circulatory vitamin-binding proteins in relation to vitamin status

3.1 Rationale

Saliva is often hailed as an attractive source of novel biomarkers for systemic disease, due to its non-invasive accessibility and diverse proteome which shares 25-30% homology to serum (198). Proteomic analysis of human saliva samples detected the presence of vitamin D-binding protein, haptocorrin, and retinol-binding protein (141). The three vitamin-binding proteins identified in saliva are also abundantly present in the circulation and have been studied in greater detail in that regard.

Vitamin D is highly hydrophobic, therefore the binding of vitamin D metabolites by vitamin D-binding protein (DBP) improves solubility and aids transit through the circulation (29). DBP has been detected in a number of biofluids in addition to serum including whole mouth saliva, and gingival crevicular fluid (199). DBP is the major circulatory transporter of vitamin D metabolites with ~85% of 25-OH-D and 1,25-$(OH)_2$-D complex to the protein (200). With such a large fraction of circulatory vitamin D metabolites being bound to DBP in the circulation, a number of studies have identified correlations between circulatory DBP concentrations and vitamin D status (200, 201).

Haptocorrin is also present in the circulation where it binds ~80% of the vitamin B_{12} present (91). The fraction of vitamin B_{12} bound to haptocorrin in the circulation is inaccessible to cells with no cellular receptor recognising the complex (79). Unique to vitamin B_{12}-binding proteins, haptocorrin binds physiologically inactive analogues of the vitamin (94). It is proposed that haptocorrin may act as a filter to remove non-physiologically active vitamin B_{12} analogues from the circulation via the asialoglycoprotein receptor (a lectin receptor expressed by hepatocytes which removes target glycoproteins from the circulation) (202). The circulatory concentration of haptocorrin is responsive to vitamin B_{12} status with low concentrations observed in patients with B_{12} deficiency and increasing in response to B_{12} supplementation (203).

As with vitamin D, vitamin A metabolites are hydrophobic (121). Solubility is improved by complexing with retinol-binding protein (RBP), which also binds transthyretin to increase the molecular weight of the complex, thus preventing loss during glomerular filtration of blood (204). A number of published studies have shown strong correlations between circulatory concentration of retinol binding-protein and vitamin A status (205, 206).

Due to the strong relationships detected between circulatory DBP, Hc, and RBP with their corresponding vitamin ligands the aim of this study was to determine the relationship between vitamin-binding protein concentrations in both saliva, and serum, with systemic markers of vitamin status. The benefits of saliva over serum as a source of biomarkers indicate that similar correlations with salivary concentrations of the proteins would be clinically beneficial. A further aim of this study was to understand the relationship between the proteins and vitamins between the two fluids which may suggest a role for the binding proteins in dietary vitamin assimilation.

3.2 Method

3.2.1 Ethical approval

Ethical approval for this study was obtained from the Biomedical Sciences, Medicine, Dentistry and Natural and Mathematical Sciences subcommittee of the King's College London Research Ethics Committee (Reference: BDM/14/15-61).

3.2.2 Participant recruitment and informed consent

14 participants were recruited from within the university, via the research recruitment email system. An individual's compatibility for the study was dependent on the following criteria;

- ≥18 years old
- In good general health (no current or recent symptoms of illness)
- No history of gastrointestinal disorder
- Not currently taking medication
- Not currently taking vitamin supplements

Informed consent was obtained from all participants.

3.2.3 Blood collection

Participants were asked to refrain from eating or drinking (other than water) for eight hours prior to blood collection, this was to ensure a baseline reading of vitamin status could be measured. Venous blood (17mL) was collected from the forearm using butterfly needle safety collection kits, into two 8.5mL Vacutainer serum separator tubes. All samples were maintained on ice until laboratory processing. Serum separator tubes were centrifuged at 2000g for ten minutes as per the manufacturer's instructions. The serum produced was then divided into aliquots of 500μL and stored at -80°C until testing.

3.2.4 Saliva collection

All samples were collected in the morning to minimise circadian variance. Resting saliva was sampled by expectoration into pre-weighed universal tubes over ten minutes. Tubes were re-weighed in order to calculate saliva secretion rate. Salivary mass was assumed equal to volume (1g=1mL). Salivary flow rate was calculated by multiplying the assumed volume of collected saliva, by the duration of collection, flow rate is expressed as mL/min. Samples were stored on ice until processing. Saliva samples were centrifuged at 13,000g for 5 minutes at 4ºC to remove any cellular or microbial debris. Samples were divided into aliquots of 200μL and stored at -80°C until testing.

3.2.5 Sample analysis

For greater detail on sample analysis please refer to Chapter 2. In brief, vitamin-binding proteins; haptocorrin, vitamin D-binding protein, and retinol-binding protein were quantified by ELISA in saliva and serum samples. Salivary total protein content was measured by BCA assay in order to load equal total protein on SDS-PAGE. Vitamin-binding proteins were detected by immuno-blot. 25-OH-D and total-vitamin B_{12} were quantified in serum by CMIA, as indicators of vitamin D and B_{12} status respectively. Retinoic acid was quantified by HPLC as an indicator of vitamin A status.

3.2.6 Statistical analysis

Correlation was analysed by linear regression with goodness of fit demonstrated by R^2-value, and corresponding p-value indicating significance from being non-zero.

3.3 Results

3.3.1 Cohort summary

No statistically significant differences were observed between male and female participants for any of the parameters in Table 3-1 when analyses by t-test. Salivary vitamin D-binding protein concentration did appear to be more variable in the female cohort (raw demographic data is presented in Appendix 1).

Table 3-1 Vitamin-binding protein concentrations and secretory outputs in saliva, and serum concentrations of vitamin-binding proteins and vitamins.

	Female	Male	Aggregate
n	6	8	14
		Mean ± SEM	
Age	30.29±2.76	31.75±4.69	30.29±2.76
Resting salivary flow rate (mL/min)	0.46±0.05	0.44±0.05	0.46±0.05
Serum [25-OH-D] (nmol/L)	40.00±8.72	40.75±8.61	40.43±5.95
Salivary [DBP] (ng/mL)	349.00±135.60	215.10±38.46	268.70±58.67
Salivary DBP output (ng/min)	205.50±96.78	88.92±17.87	138.90±43.57
Serum [DBP] (μg/mL)	376.60±53.17	321.70±40.52	345.20±32.06
Serum [total-B_{12}] (ng/L)	246.50±34.53	279.50±35.78	265.40 ±24.72
Salivary [Hc] (pg/mL)	34.78±9.05	30.14±4.77	31.70±4.77
Salivary Hc output (pg/min)	69.39±15.23	34.18±7.92	49.27±9.00
Serum [Hc] (pg/mL)	545.40±171.6	443.10±101.20	486.90±90.55
Serum [retinol] (μmol/L)	1.99±0.15	2.14±0.15	2.08±0.10
Salivary [RBP] (μg/mL)	1.44±0.10	1.44±0.11	1.44±0.08
Salivary RBP output (μg/min)	0.51±0.07	0.79±0.14	0.67±0.09
Serum [RBP] (μg/mL)	95.17±1.60	90.73±3.49	96.63±2.13

3.3.2 Salivary vitamin-binding proteins

Figure 3-1 confirms by immuno-blot, the presence of vitamin D-binding protein, haptocorrin, and retinol-binding protein in resting whole mouth saliva previously demonstrated by proteomic analysis (141). Samples were normalised by total protein concentration prior to electrophoresis to account for interpersonal variation. A number of samples have particularly strong signals in comparison to the remainder, for example C-i, J-i, and E-ii.

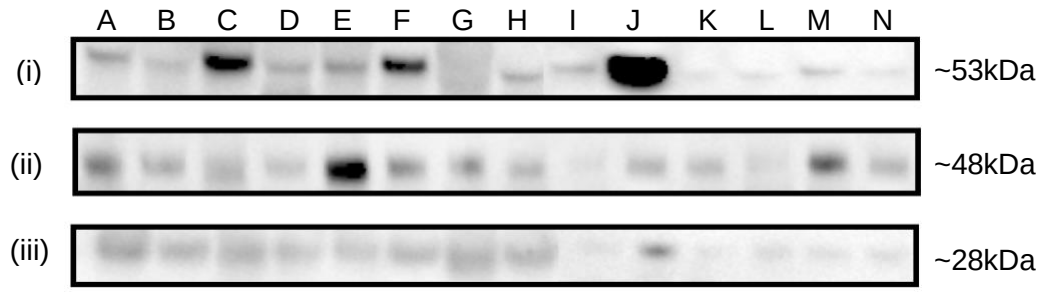

Figure 3-1 Detection of salivary vitamin-binding proteins by immunoblot.
Resting unstimulated whole mouth saliva samples from 14 study participants (A-N). Blots immunoprobed for (i) vitamin D-binding protein, (ii) haptocorrin, and (iii) retinol binding protein**.**

3.3.3 Vitamin D and vitamin D-binding protein

Figure 3-2 represents the distribution of vitamin D-status in the study cohort (raw values can be found in Appendix 2. 25-hydroxyvitamin D (25-OH-D) is a robust and stable marker of vitamin D status (207). Reference ranges vary; however, serum 25-OH-D concentrations are commonly divided into the following three categories of sufficient (>50nmol/L), insufficient (30-50nmol/L), and deficient (<30nmol/L) (208). The incidence of deficient and insufficient participants in this cohort although striking for a group of healthy individuals has been previously reported by a number of large-scale epidemiological studies (44).

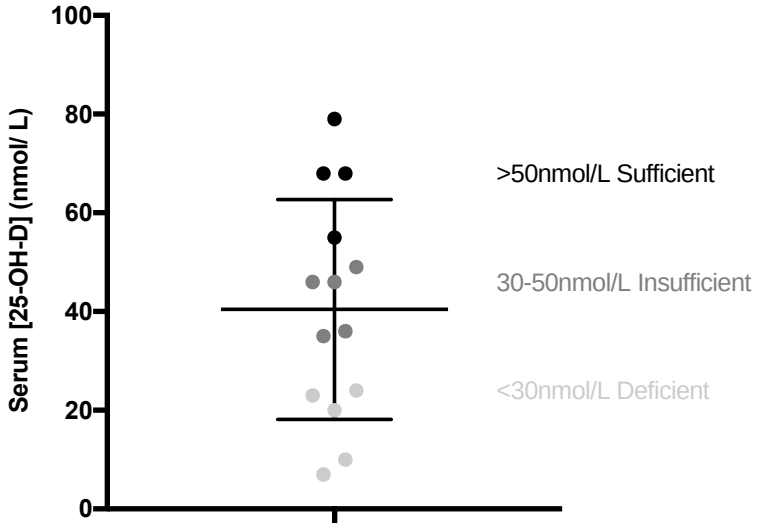

Figure 3-2 Distribution of vitamin D status in the study cohort.
Determined by serum 25-OH-D concentration (nmol/L). Grey-scale of data points is descriptive of clinical categorisation of vitamin D status.

Figure 3-3 demonstrates the correlation analysis of serum, and salivary vitamin D-binding protein (DBP) concentration, and salivary DBP output (raw value can be found in Appendix 2). Serum DBP concentration (left Y-axis) and 25-OH-D showed the

strongest relationship although this is not quite significant (R^2 0.26, p 0.06). Salivary DBP, concentration and output shared no clear relationship with vitamin D status. Furthermore, the serum concentration of DBP is observed here approximately 1000-fold higher than that of saliva, which is not uncommon for proteins shared between the two biofluids (1).

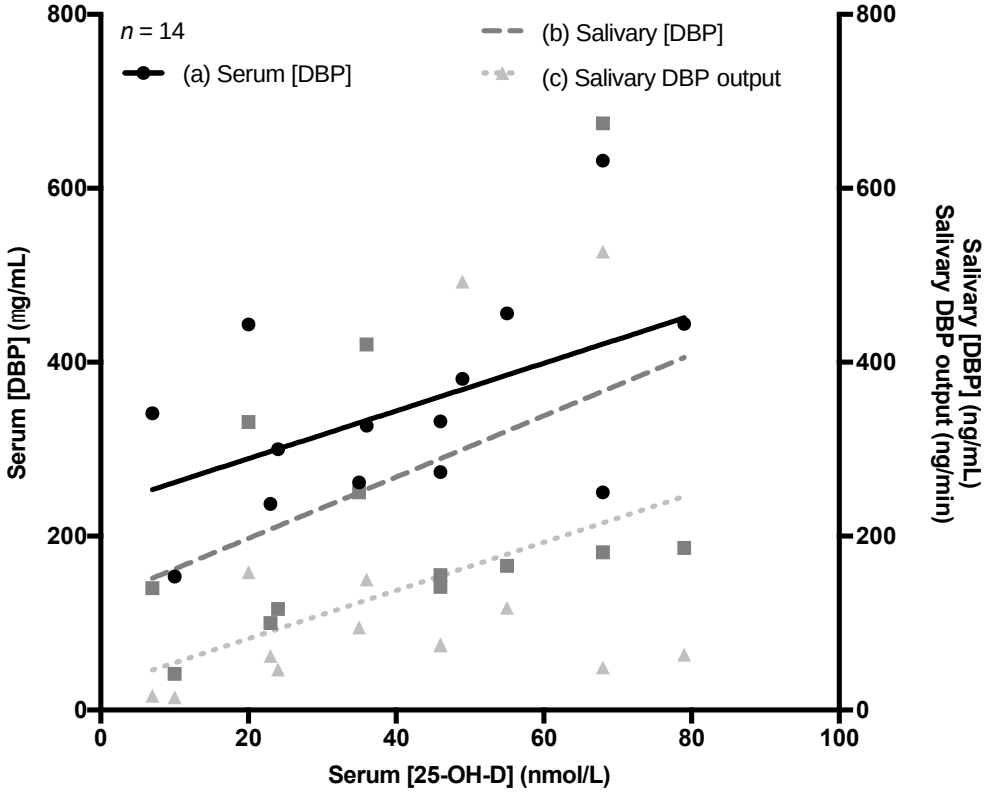

Figure 3-3 The relationship between 25-OH-D, and circulatory and salivary vitamin D-binding protein.
Individual values and line of best fit presented. Correlation analysis of serum and salivary vitamin D-binding protein (DBP) concentration and output against the commonly used marker of vitamin D status, 25-hydroxyvitamin D (nmol/L). (a) Serum DBP concentration (µg/mL) (left axis) R^2 0.26 (p 0.06). (b) Salivary DBP concentration (ng/mL) (right axis) R^2 0.11 (p 0.25). (c) Salivary DBP output (ng/min) (right axis) R^2 0.14 (p 0.18).

A positive relationship was observed between DBP concentration in the two biofluids (Figure 3-4) (raw values can be found in Appendix 2) of R^2 0.33 (p 0.03) suggesting expression is regulated in the same manner, or salivary DBP is a serum contaminant. The relationship with salivary DBP output was even stronger with R^2 0.45 (p 0.01).

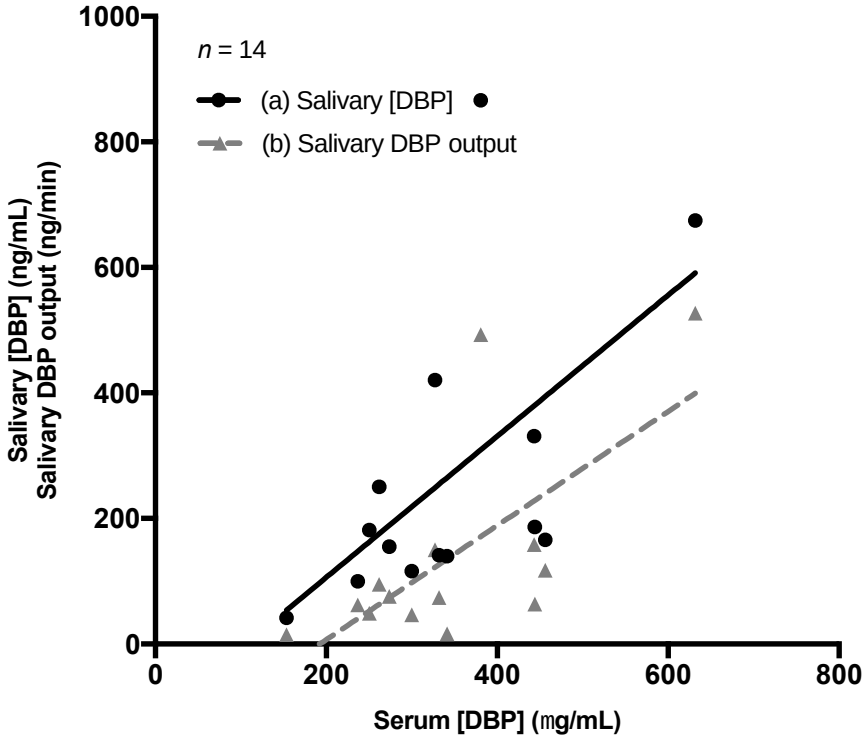

Figure 3-4 The relationship between serum vitamin D-binding protein concentration and salivary concentration and output
Individual values and line of best fit presented. Correlation analysis of salivary vitamin D-binding protein (DBP) concentration and output against serum DBP concentration (µg/mL). (a) Salivary DBP concentration (ng/mL) R^2 0.33 (p 0.03). (b) Salivary DBP output (ng/min) R^2 0.45 (p 0.01).

3.3.4 Vitamin B_{12} and haptocorrin

Figure 3-5 illustrates the distribution of vitamin B_{12} status in the study cohort. Fasting serum samples were used to measure total-vitamin B_{12} concentration. Three study participants were below the lower threshold of healthy vitamin B_{12} status. The commonly

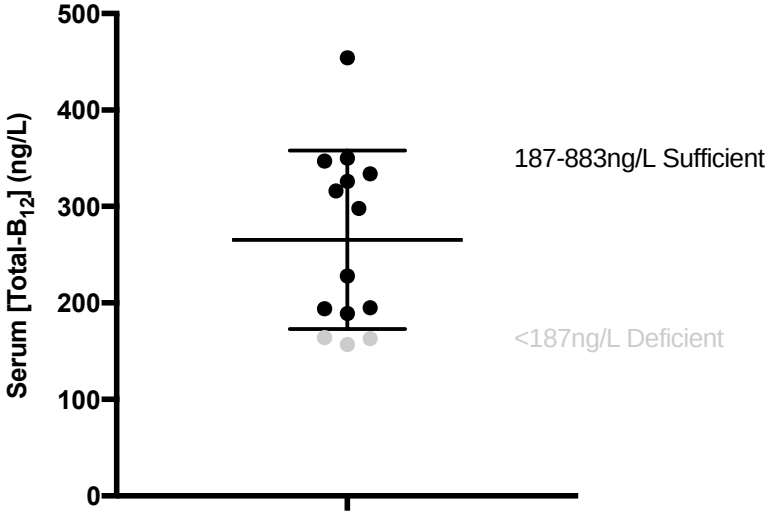

Figure 3-5 Distribution of vitamin B_{12} status in the study cohort.
Determined by total serum vitamin B_{12}. Grey points indicate a value outside of the "healthy' range of 187-883 ng/L.

used reference range of 187-883ng/L represented with grey points for individuals outside of that range. This reference range for diagnostic purposes is not infallible, with some individuals within this range, reported to manifest signs of deficiency, however for the purpose of correlation in this study, total-vitamin B_{12} was deemed a valid marker.

Figure 3-6 represents the correlation analysis between total-vitamin B_{12} concentration, and that of serum and salivary concentrations of haptocorrin as determined be ELISA. The resting salivary output of haptocorrin is also shown, this considers the salivary flow rate and can represent the non-static nature of salivary composition. A significant correlation was observed between serum haptocorrin concentration and that of total-B_{12} with an R^2 value of 0.50 ($p < 0.01$). Salivary haptocorrin concentration, and output demonstrated no significant relationship to systemic vitamin B_{12} status.

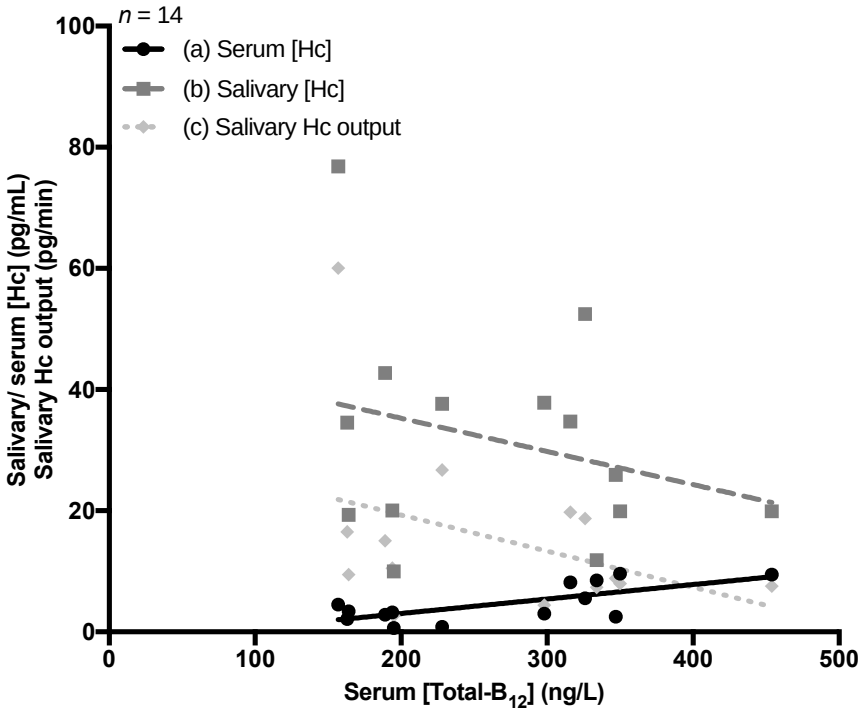

Figure 3-6 The relationship between vitamin B_{12}, and circulatory and salivary haptocorrin.
Individual values and line of best fit presented. Correlation analysis of serum and salivary haptocorrin (Hc) concentration and salivary output against total serum vitamin B_{12} (ng/L), a commonly employed marker of vitamin B_{12} status. (a) Serum Hc concentration(pg/mL) R^2 0.50 ($p < 0.01$). (b) Salivary Hc concentration (pg/mL) R^2 0.08 (p 0.33). (c) Salivary Hc output (pg/min) R^2 0.15 (p 0.18).

No significant relationship was found between the concentration of haptocorrin in serum or saliva, or between serum concentration and salivary Hc output (Figure 3-7).

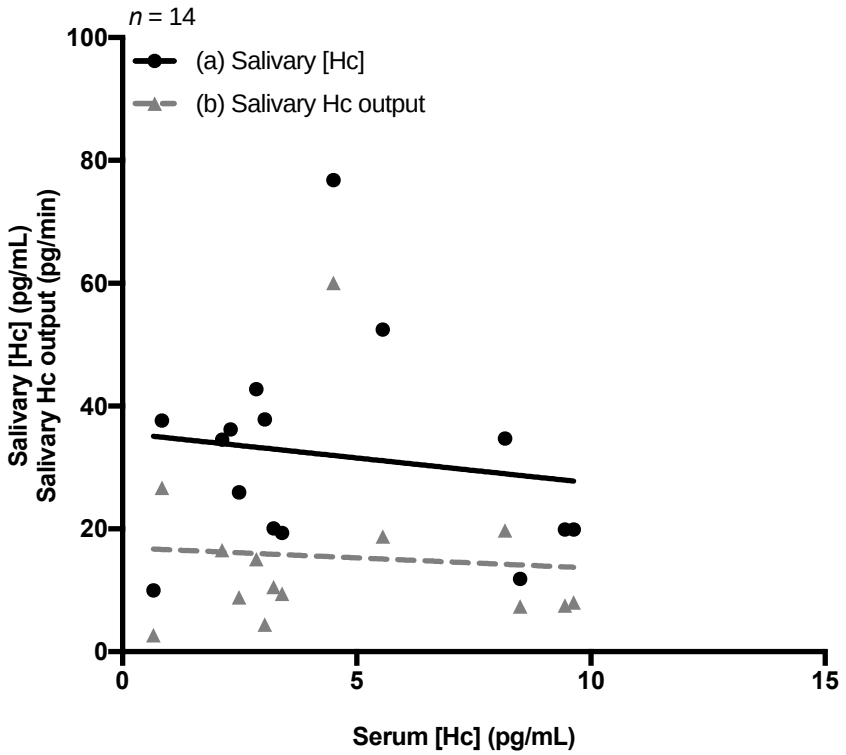

Figure 3-7 The relationship between serum haptocorrin concentration and salivary concentration and output

Individual values and line of best fit presented. Correlation analysis of salivary haptocorrin (Hc) concentration and output against serum Hc concentration (pg/mL). (a) Salivary Hc concentration (pg/mL) R^2 0.02 (p 0.61). (b) Salivary Hc output (pg/min) R^2 <0.01 (p 0.81).

3.3.5 Vitamin A and retinol-binding protein

Figure 3-8 shows the distribution of serum retinol concentration, which was used as a marker of vitamin A status (raw data can be found in Appendix 4). A commonly used reference range for retinol is 1.4-3.84μmol/L for which all participants were within.

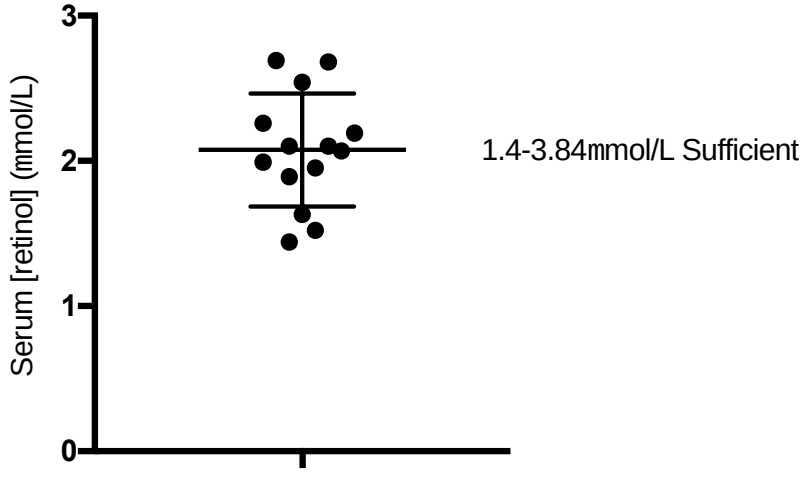

Figure 3-8 Distribution of vitamin A status in the study cohort.

Determined by circulatory retinol concentration. All participants fell within a "healthy" range of 1.4-3.84μmol/L.

Figure 3-9 demonstrates a significant relationship between retinol, and serum retinol-binding protein (RBP) concentration with an R^2 of 0.48 ($p < 0.01$) (left Y-axis). No clear relationship was seen between retinol and salivary RBP concentration or output. Salivary RBP concentration was demonstrated to be 100-fold less than that of serum.

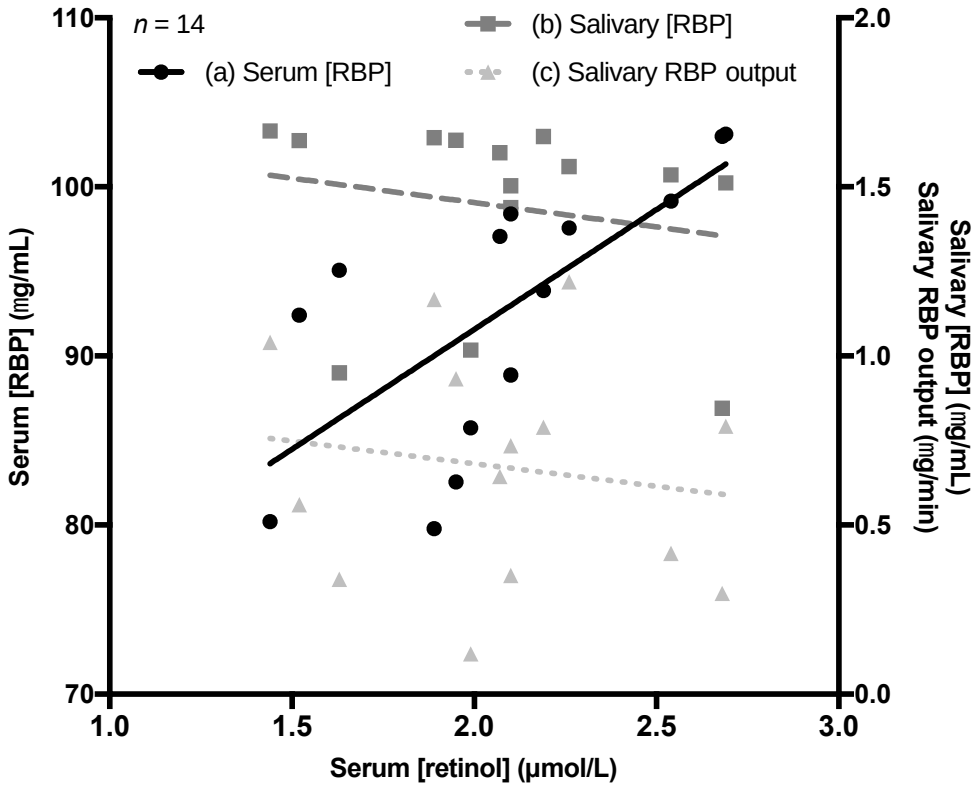

Figure 3-9 The relationship between vitamin A, and circulatory and salivary retinol-binding protein.
Individual values and line of best fit presented. Correlation analysis of serum and salivary retinol-binding protein (RBP) concentration and output against circulatory retinol (µmol/L). (a) Serum RBP concentration (µg/mL) (left axis) R^2 0.48 ($p < 0.01$). (b) Salivary RBP concentration (µg/mL) (right axis) R^2 0.04 (p 0.50). (c) Salivary RBP output (µg/min) (right axis) R^2 0.02 (p 0.60).

No significant relationship was seen between serum retinol-binding protein concentrations, and those in saliva, or salivary retinol-binding protein output (Figure 3-10).

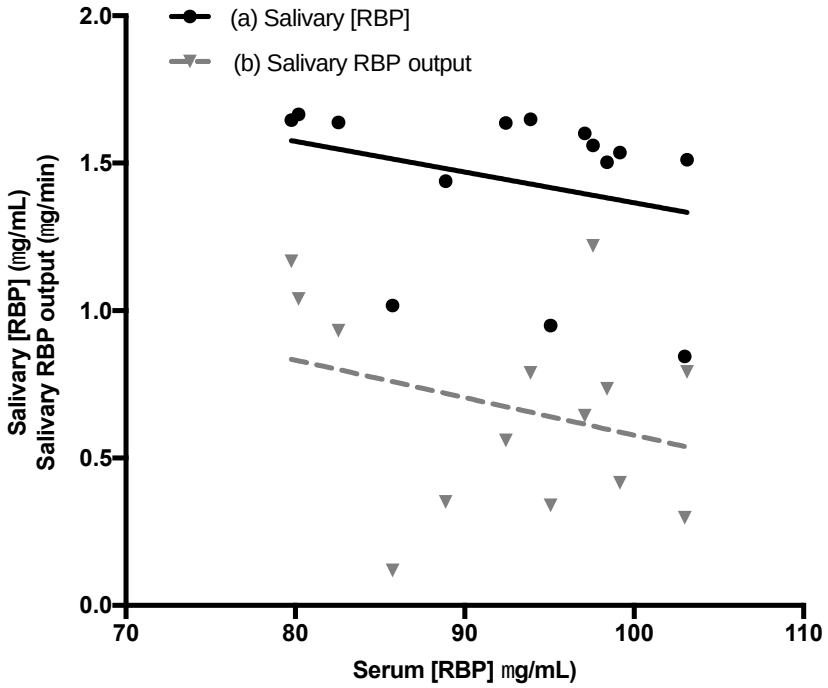

Figure 3-10 The relationship between serum retinol-binding protein concentration and salivary concentration and output

Individual values and line of best fit presented. Correlation analysis of salivary retinol-binding protein (RBP) concentration and output against serum RBP concentration (µg/mL). (a) Salivary RBP concentration (µg/mL) R^2 0.09 (p 0.31). (b) Salivary RBP output (µg/min) R^2 0.09 (p 0.30).

Figure 3-11 demonstrates a statistically significant inverse association between age and salivary retinol-binding protein concentration and output. This differed from salivary flow rate, and total salivary protein concentration which showed no significant relationship with age of participants. No other parameter in this study demonstrated a significant correlation with age (Appendix 5).

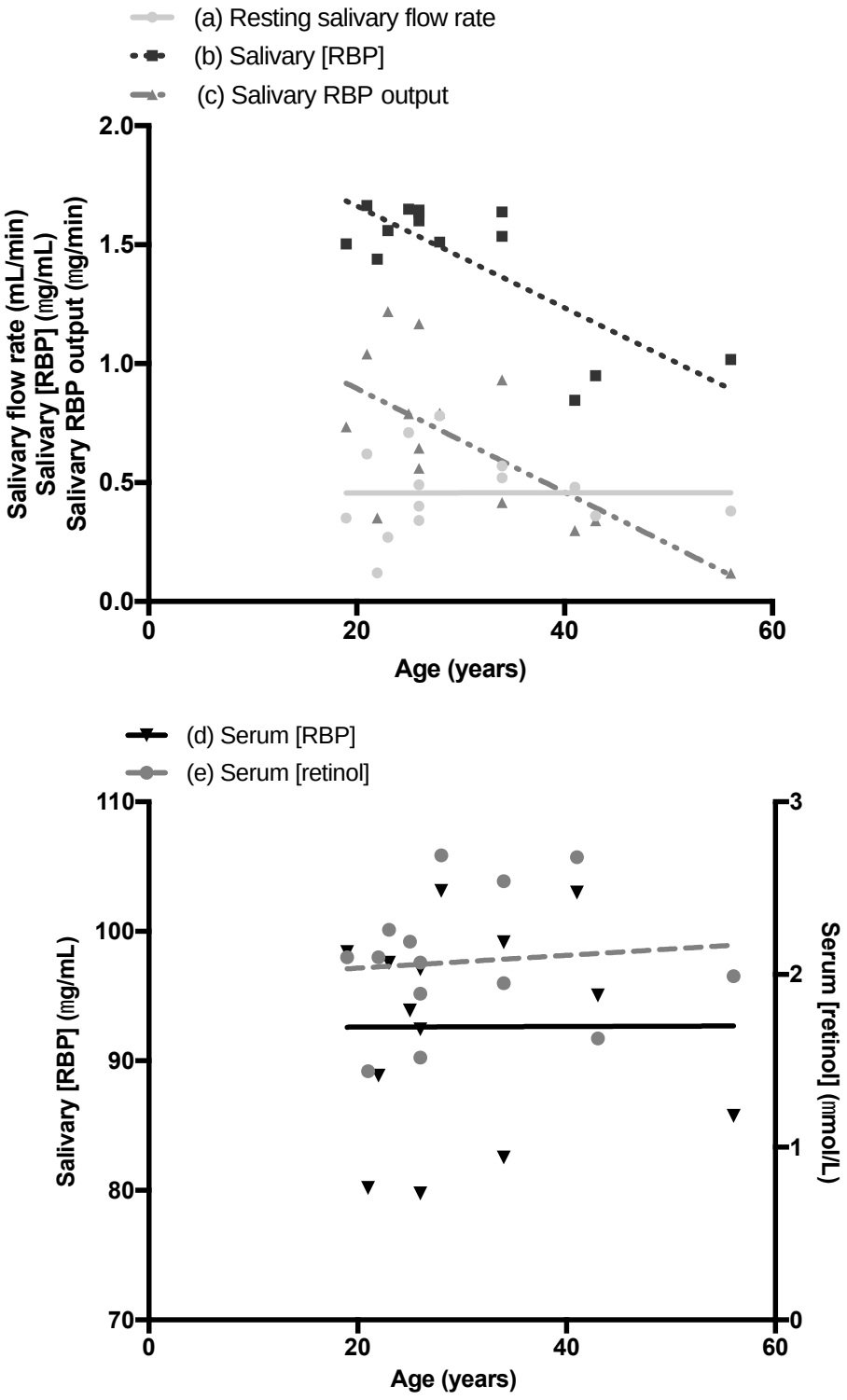

Figure 3-11 Age-associated differences in salivary retinol-binding protein concentration and output.

Individual values and line of best fit presented. Correlation analysis of participant age (years) and (a) resting salivary flow rate (mL/min) R^2 <0.01 (p 0.99). (b) Salivary retinol-binding protein (RBP) concentration (µg/mL) R^2 0.61 (p <0.01). (c) Salivary RBP output (µg/min) R^2 0.44 (p 0.01). (d) Serum RBP concentration (µg/mL) R^2 <0.01 (p 0.99). (e) Serum retinol (µmol/L) concentration R^2 0.12 (p 0.74).

3.4 Discussion

Whole mouth saliva is a complex easily assessible biofluid, with a catalogue of over two thousand protein constituents (141). Approximately 27% of the salivary proteome is shared with that of plasma (198). Consequently; the diagnostic potential of saliva for systemic diseases is a popular area of research. The work in this initial chapter examined the relationship between commonly used clinical markers of vitamin status, and vitamin-binding proteins present in both saliva and blood.

No statistically significant differences were noticed between male and female participants in this study; however, greater variance was seen in vitamin D-binding protein output in the female cohort. There is some evidence in the literature to suggest the use of hormonal contraceptives may influence plasma concentrations of vitamin D metabolites and DBP (209). Although our data does not suggest this for serum, salivary gland expression of DBP may be influenced by hormonal contraceptives, which has been demonstrated previously for other salivary constituents (210, 211). Future studies of a similar nature should take the use of hormonal contraceptives into account.

The distribution of serum 25-OH-D concentrations, and the incidence of insufficiency and deficiency in this study is quite striking, though not dissimilar to published studies which have reported approximate hypovitaminosis D prevalence of 50% in British adults (212). In this study, salivary DBP concentration did not correlate as well as serum DBP to circulatory 25-OH-D, however salivary and serum DBP did correlation to each other, with approximately 2000-fold difference in concentration. Circulatory DBP is largely expressed by hepatocytes, however the source of salivary DBP remains unclear (213). One challenge in studying dietary vitamin D uptake arises from endogenous synthesis. Dissimilar to the other twelve vitamins, vitamin D can by synthesised in the dermis by UV-B irradiation of 7-dehydrocholesterol, which yields pre-vitamin D, and over time at body temperature this is converted to vitamin D (22). The trend between salivary and serum DBP concentrations could suggest that it enters saliva from the circulation or salivary gland expression of DBP is under the same regulation as the systemic sources of the protein.

The importance of salivary and gastric haptocorrin (Hc) has long been suggested for the early assimilation pathway of biologically active cobalamin metabolites (79, 214, 215). In the digestive tract, Hc offers two key roles, firstly in protecting all corrinoids (cobalamin and its analogues) from hydrolysis in the acidic milieu of the stomach (79). Secondly, Hc acts as a selective sieve by promiscuously binding corrinoids, and not dissociating from

biologically inactive analogues. As a consequence, these complexes are not absorbed in the gastrointestinal tract and are excreted (93, 216). The results in this chapter suggest salivary haptocorrin did not correlate with total serum vitamin B_{12}, however serum Hc concentration did. The correlation between serum Hc concentration and total-B_{12} is not unexpected with approximately 70-80% of circulatory B_{12} being bound to haptocorrin (217). The lack of relationship observed between salivary and serum Hc concentrations suggests it is actively added to saliva and is not simply passive exudation from the circulation. A surprising observation from this data however, is the similarity in Hc concentrations between saliva and serum samples, often proteins which feature in the proteomes of both biofluids are typically a thousand-fold lower in the former, the concentrations in the two fluids did not correlate significantly (138).

Retinol-binding protein, occasionally referred to as retinol-binding protein 4, is the sole circulatory transporter with specificity for vitamin A and its metabolites. Expression of RBP is predominantly undertaken by hepatocytes (218). Serum RBP concentration correlates well with serum retinol, however salivary RBP concentration does not. As with the lack of correlation identified with the other two vitamin-binding proteins present in saliva, the results in this chapter highlight questions on the regulation and source of these proteins in the oral cavity. Furthermore, an inverse association was seen between salivary RBP and age, it is possibly a consequence of the small sample size or altered expression of RBP by salivary glands with age (219).

The results in this chapter demonstrate no clear correlation between salivary vitamin-binding protein concentration or output and corresponding markers of vitamin status. These data do raise questions regarding the source, and indeed function of these proteins within the oral cavity.

Chapter 4 Characterisation of vitamin-binding proteins in the context of the oral cavity

4.1 Rationale

The ability of saliva to facilitate the diverse functions of the oral cavity is, in part, due to multifunctional properties of the proteins in saliva (220). In addition, the composition, and properties of whole mouth saliva demonstrate circadian variance (147, 221). Salivary haptocorrin for example, is capable of binding both physiologically active, and inactive forms of vitamin B_{12} which occurs upon liberation from the food matrix in the upper digestive tract. The binding affinity of haptocorrin for cobalamin is 50- fold greater than other cobalamin-binding proteins in the GI lumen and the highly glycosylated structure of haptocorrin is thought to protect the vitamin from hydrolysis in the acidic milieu of the stomach lumen (80). Haptocorrin is then cleaved by pancreatic proteases, allowing binding by intrinsic factor, which facilitates absorption across the brush boarder (222). Most of the literature supporting the hypothesised role of haptocorrin in vitamin assimilation, is based on biochemical and biophysical studies, yet do not consider their physiological source, or concentration of the vitamin-binding proteins.

Since whole mouth saliva is a mixture of glandular saliva and serum exudate from the gingival margin, it should be established if vitamin-binding proteins are made and actively secreted by salivary glands or simply a leakage product from blood. An assimilatory role for the other vitamin-binding proteins such as vitamin D-binding protein and retinol-binding protein has yet to be proposed. If assimilation of vitamins is the main role of the vitamin-binding proteins it might be expected that they would be synthesised by the parotid glands as these are the most up-regulated when salivary flow is stimulated by taste and mastication stimuli (1).

4.2 Aims

To measure salivary vitamin-binding protein concentration and output at rest and during stimulated flow, to understand actual amounts of binding proteins. The secondary aim of this study was to quantify vitamin-binding protein concentrations in different saliva types to suggest their origin in whole mouth saliva.

4.3 Method

4.3.1 Ethical approval

Ethical approval for this study was obtained from the Biomedical Sciences, Medicine, Dentistry and Natural and Mathematical Sciences subcommittee of the King's College London Research Ethics Committee (Reference: BDM/14/15-61), and subsequent amendment granted 24th January 2018.

4.3.2 Participant recruitment and informed consent

Potential participants were recruited from within the university, via the research recruitment email system. An individual's compatibility for the study was dependent on the following criteria;

- ≥18 years old
- In good general health (no current or recent symptoms of illness)
- No history of gastrointestinal disorder
- Not currently taking medication
- Not currently taking vitamin supplements

Informed consent was obtained from all participants.

4.3.3 Comparing resting and mastication stimulated saliva

Five female, and five male "healthy" participants were recruited. "Healthy" being defined as no current or recent symptoms of illness, and not currently taking prescription medication. Participants also declared no recent vitamin supplement use.

Resting saliva was sampled by expectoration into pre-weighed universal tubes over five minutes. Tubes were re-weighed to calculate saliva secretion rate. With 1mL volume assumed for 1g of weight. There are a number of commonly employed methods for saliva stimulation including mastication; typically, of a flavourless gum, citric acid or by stimulation by other tastants, or olfaction (223). Mastication of a flavourless gum was selected to best replicate the stimulation which would occur consuming foods containing the three vitamins of interest. Participants were then asked to chew a flavourless paraffin gum until they reported an increase in the salivary flow rate, then swallow the saliva, and while continuing to chew expectorate for a further five minutes into another pre-weighed universal tube, the flow rate was calculated, and samples were maintained on ice until processing.

4.3.4 Glandular vitamin-binding protein contributions

One female and one male participant (30yo and 26yo, respectively) were recruited following the same eligibility criteria stated above. Unstimulated whole mouth saliva (WMS) was collected by expectoration into a pre-weighed universal tube for a duration of three minutes. Resting parotid saliva was collected by attaching Lashley cups (King's Medical Science, U.K.) to both the right and left papillae of the Stensen's ducts (parotid ducts) which are located in the buccal mucosa, adjacent to the second superior molar (refer to Figure 4-1).

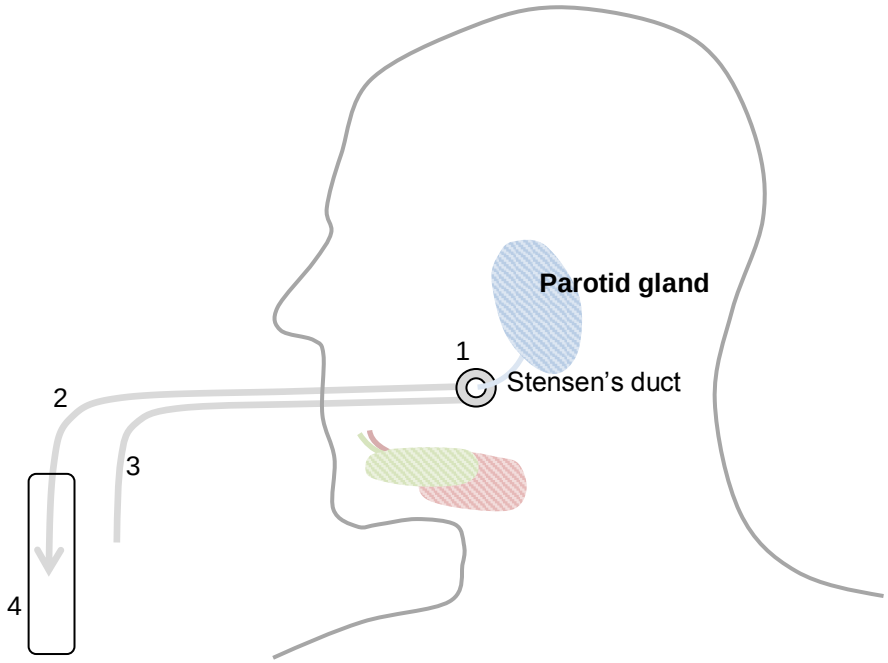

Figure 4-1 Collection of parotid gland secretion by the Lashley cup method

(1) Suction cup which attaches to the Stensen's duct papillae
(2) Tube carrying glandular secretion
(3) Tube providing suction to hold the cup in place and prevent contamination or loss of secretion
(4) Universal tube for saliva collection

The mouth was rinsed gently for thirty seconds with water. Unstimulated parotid saliva was collected for ten minutes, at the same time whole mouth saliva was collected by expectoration into a pre-weighed universal tube, this will be referred to as whole mouth saliva (non-parotid) (WMS (NP)). Saliva was stimulated by mastication, using flavourless gum. Right and left parotid saliva and WMS(NP) were collected for three minutes when increase flow was noticed. Finally, upon removal of the Lashley cups, stimulated whole mouth saliva was collected by expectoration for a duration of three minutes. All samples were reweighed post collection in order to calculate flow rate and stored on ice until processing.

Due to the small sample size, and high degree of interpersonal variation within sample types, each participant's sample set was expressed as a percentage of resting WMS concentration.

4.3.5 Saliva analysis

For more detail on sample analysis please refer to Chapter 2 materials and methods. In brief, saliva samples were centrifuged at 13,000g for five minutes at 4°C. Vitamin D-binding protein, haptocorrin, and retinol-binding protein were quantified in all sample types by ELISA. Salivary total protein content was measured by BCA assay, haptocorrin was also visualised by western blot.

4.3.6 Statistical analysis

Distribution of the data was determined by D'Agostino & Pearson normality test; all data were normally distributed. Paired t-tests were used for the comparison of resting and stimulated samples.

4.4 Results

4.4.1 Comparing resting and stimulated whole mouth saliva

Table 4-1 demonstrates the mean increase in salivary flow rate upon stimulation, this was coupled with a small yet significant decrease in total protein concentration. The total protein output which is the flow multiplied by concentration however, increased significantly upon mastication stimulation. Gender demonstrated no effect on any of the parameters tested, excluding resting RBP concentration (please refer to Appendix 7).

Table 4-1 Flow rate and total protein changes in resting and chew-stimulated whole mouth saliva

***n*=10**

	Mean±SEM		
Age	33.4±2.45		
	Resting	**Stimulated**	
Flow rate (mL/min)	0.70±0.09	1.95±0.29	p <0.01
Total protein concentration (mg/mL)	1.66±0.16	1.29±0.11	p <0.05
Total protein output (mg/mL)	1.09±0.08	2.35±0.31	p <0.01

Paired t-test results presented comparing resting to stimulated

4.4.2 Vitamin D-binding protein

For the majority of participants in this study (eight out of ten), salivary vitamin D-binding protein was lower in concentration in stimulated whole mouth saliva compared to stimulated (Figure 4-2 (a)). Two participants demonstrated an increase in DBP concentration with mastication. No significant difference was observed between resting and stimulated DBP concentration for the cohort as a whole assessed by paired t-test. Figure 4-2 (b) presents the change in salivary DBP output between resting and stimulated flow and considers the increase in flow rate with mastication. Whole mouth DBP output increased significantly for all participants when assessed by paired t-test (a table of raw values can be found in Appendix 8).

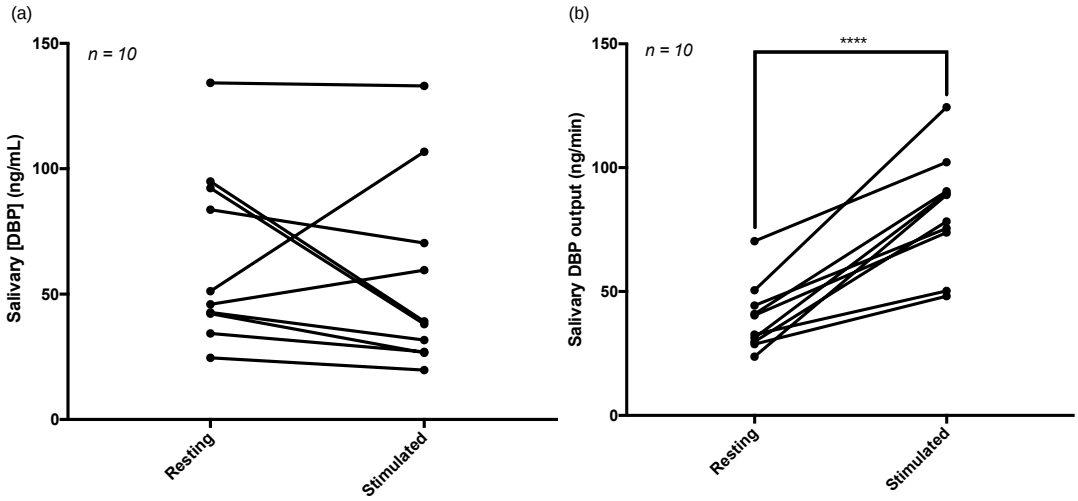

Figure 4-2 Salivary vitamin D-binding protein concentration and output at resting and stimulated flow measured by ELISA.

(a) Subject paired salivary vitamin D-binding protein concentration at resting flow and with mastication stimulation
(b) Subject paired salivary vitamin D-binding protein output (flow x concentration) at rest and mastication stimulated (**** = $p < 0.0001$). Analysed by paired t-test

To understand which of the major salivary glands secrete most of DBP, Figure 4-3 presents salivary vitamin D-binding protein concentration (a), and output (b), for two participants (one male and one female) expressed as a percentage of each individuals WMS concentration or output respectively. Normalisation was undertaken in order to account for inter-personal variation in DBP concentration. Whole mouth saliva (WMS), right and left parotid, and whole mouth saliva excluding parotid (WMS(NP)) were collected at resting and mastication stimulated flow. The first observation to make from these data, is the difference between the two participants, with participant two having greater DBP concentrations and outputs in all saliva categories. Complimentary to Figure 4-2 (a), stimulated DBP concentration both increased and decreased marginally from the resting concentration between the two participants. The mean across participants

however, remained comparable to the resting concentration. Left and right parotid saliva from both participants demonstrated a decrease in concentration upon stimulation, though considering the large increase in flow rate (please refer to Appendix 11) with stimulation, the DBP output from the two parotid glands (Figure 4-3 (b)) remained consistent. The greatest source of DBP in stimulated whole mouth saliva was not parotid in origin as indicated by WMS(NP) Figure 4-3 (b), this is most acutely apparent in WMS(NP) output (b) (raw data is summarised in Appendix 11).

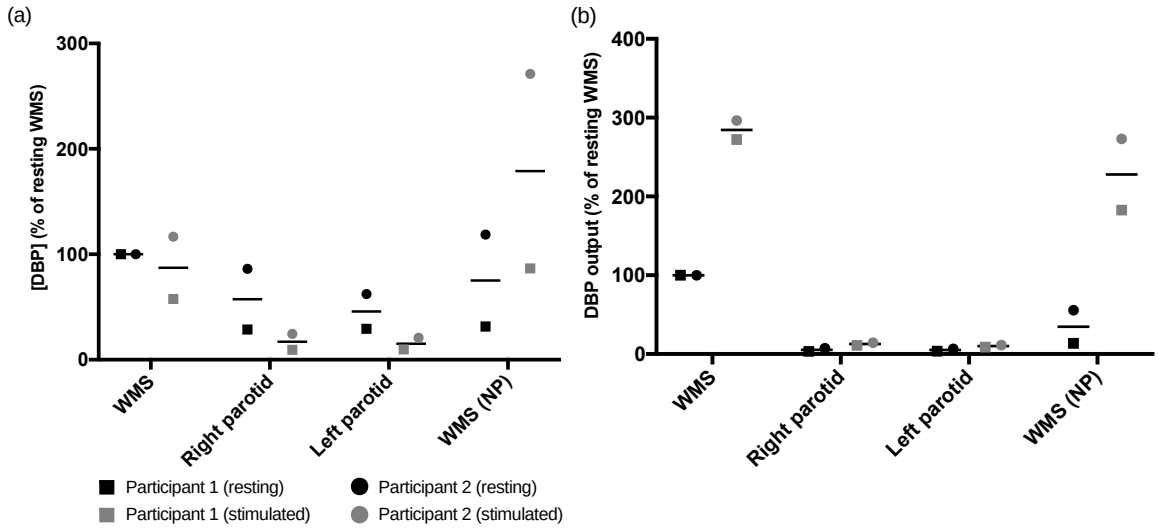

Figure 4-3 Relative salivary vitamin D-binding protein concentration and output by saliva type, measured by ELISA.

Individual measurements expressed as a percentage of resting WMS, means are also presented

(a) Salivary vitamin D-binding protein concentration of two participants at resting and mastication stimulated flow for whole mouth saliva (WMS), left and right parotid glands, and whole mouth saliva excluding parotid (WMS(NP)), each sample expressed as a percentage of WMS.

(b) Salivary vitamin D-binding protein output for two participants at resting and mastication stimulated flow for whole mouth saliva (WMS), left and right parotid glands, and whole mouth saliva excluding parotid (WMS(NP)), each sample set expressed as a percentage of WMS.

4.4.3 Haptocorrin

When visualised by western blot (Figure 4-4), the haptocorrin resting (R) band intensity is greater for most (8 participants) of the 10 participants (A-J) than in saliva stimulated by mastication (S). Equal volumes of each sample were loaded onto the SDS-PAGE gel.

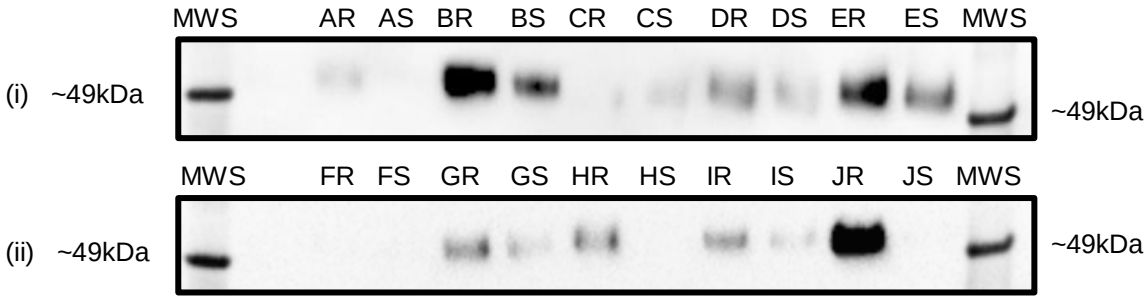

Figure 4-4 Immunoblot of salivary haptocorrin, at resting and stimulated flow.
Participants A-J, illustrating the difference in haptocorrin concentration, when equal volume is loaded between resting (R) and mastication stimulated (S) whole mouth saliva samples. (MWS – molecular weight standard)

These results were later confirmed by ELISA and are represented in Figure 4-5 (a), which demonstrates a highly significant decrease in salivary haptocorrin concentration between resting and stimulated saliva for the same 10 participants. Data presented a normal distribution and therefore were analysed by paired t-test. Figure 4-5 (b) represents salivary haptocorrin output of the 10 participants, calculated by multiplying haptocorrin concentration by the salivary flow rate. Salivary haptocorrin output increased significantly with mastication stimulation. (raw values can be found in Appendix 9).

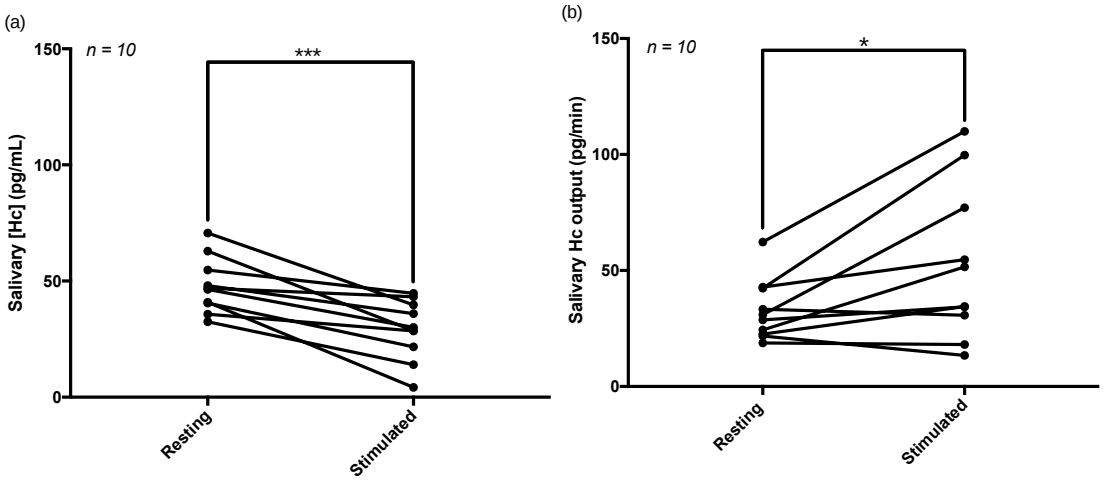

Figure 4-5 Salivary haptocorrin concentration and output at resting and stimulated flow, measured by ELISA.

(a) Subject paired haptocorrin concentration at resting flow and with mastication stimulation (*** = $p < 0.001$).
(b) Subject paired salivary haptocorrin output at resting flow and mastication stimulated (* = $p < 0.05$), analysed by paired t-test.

Hc concentration decreased but increased in output for the two participants represented in Figure 4-6. Both participants demonstrated higher concentrations in resting right and left parotid secretions compared to stimulated. The trend in WMS(NP) Hc concentration (Figure 4-6 (a)) between rest and stimulated flow was inverse between participants. Hc output for both participants, and all saliva categories increased with stimulation (Figure 4-6 (b)) and demonstrated the majority of Hc output was not parotid in origin (raw data is presented in Appendix 11).

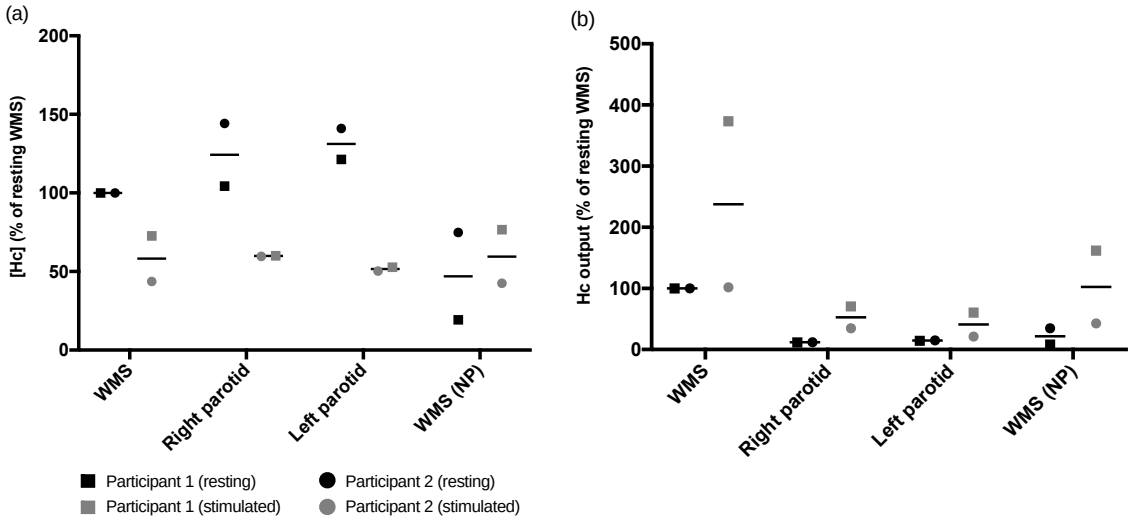

Figure 4-6 Relative salivary haptocorrin concentration and output by saliva type, measured by ELISA.

Individual measurements expressed as a percentage of resting WMS, means are also presented

(c) Salivary haptocorrin concentration of two participants at resting and mastication stimulated flow for whole mouth saliva (WMS), left and right parotid glands, and whole mouth saliva excluding parotid (WMS(NP)), each sample set expressed as a percentage of WMS.

(d) Salivary haptocorrin output for two participants at resting and mastication stimulated flow for whole mouth saliva (WMS), left and right parotid glands, and whole mouth saliva excluding parotid (WMS(NP)), each sample set expressed as a percentage of WMS.

4.4.4 Retinol-binding protein

With mastication-stimulated flow, retinol-binding protein concentration decreased in 60% of participants when compared to resting concentration (Figure 4-7 (a)), though no significant difference was detected between the categories when assessed by paired t-test. A significant increase was found in RBP output (Figure 4-7 (b)); however, this increase was not consistent for all participants.

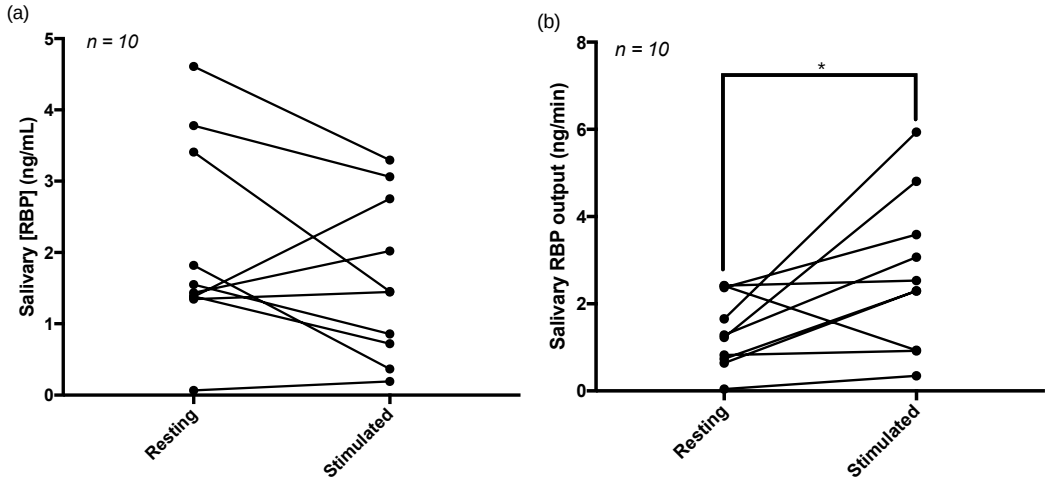

Figure 4-7 Salivary retinol-binding protein concentration and output at resting and stimulated flow, measured by ELISA.

(a) Subject paired RBP concentration at resting flow and with mastication stimulation
(b) Subject paired salivary RBP output at resting flow and mastication stimulated (* = $p < 0.05$), analysed by paired t-test.

RBP concentration and output both increased with stimulation in Figure 4-8 (raw values can be found in Appendix 10). The trend in RBP concentration by the parotid glands at rest and stimulated flow is unclear, with participant 1 demonstrating an increased RBP concentration with stimulation from the right parotid gland alone (Figure 4-8). RBP output between parotid and non-parotid saliva demonstrate modest increases with mastication (raw data is presented in Appendix 11).

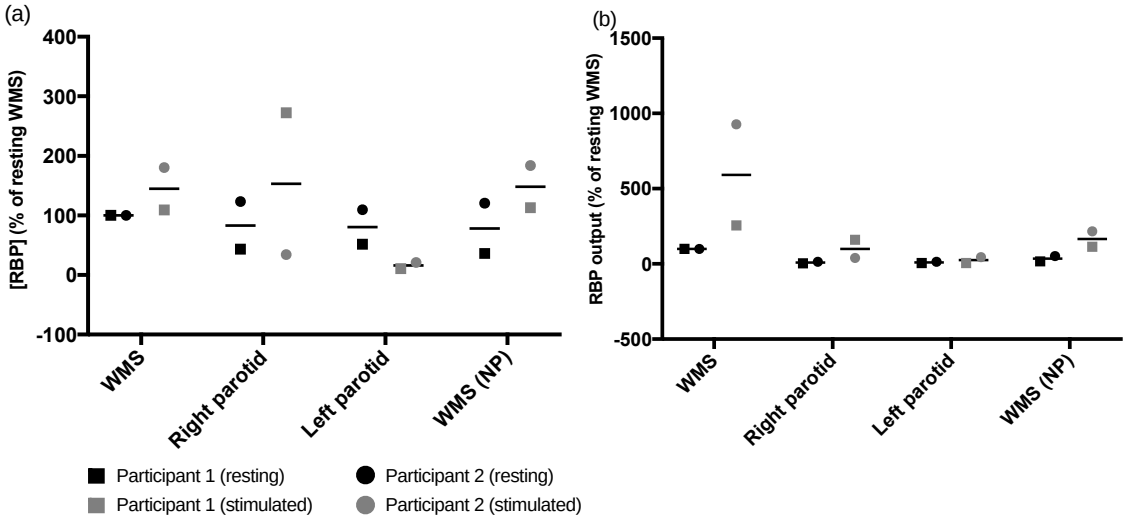

Figure 4-8 Relative salivary retinol-binding protein concentration and output by saliva type, measured by ELISA.

Individual measurements expressed as a percentage of resting WMS, means are also presented

(a) Salivary retinol-binding protein concentration of two participants at resting and mastication stimulated flow for whole mouth saliva (WMS), left and right parotid glands, and whole mouth saliva excluding parotid (WMS(NP)), each sample set expressed as a percentage of WMS.

(b) Salivary RBP output for two participants at resting and mastication stimulated flow for whole mouth saliva (WMS), left and right parotid glands, and whole mouth saliva excluding parotid (WMS(NP)), each sample set expressed as a percentage of WMS.

4.5 Discussion

Results in this section indicate all three vitamin-binding proteins studied were detectible components of salivary gland secretions. Examining a salivary protein concentration alone is insufficient as concentration is dependent on salivary flow which is variable, even within a single subject, and expresses circadian variance (224, 225). All three proteins increased significantly in output when salivary flow was upregulated by mastication. Of the vitamin-binding proteins focused on in this study, salivary haptocorrin has been the most reported in the literature to contribute to the assimilation pathway of its vitamin ligand (79). Haptocorrin is considered to play a role in protecting vitamin B_{12} from hydrolysis during gastric processing of food; however, understanding the output of salivary haptocorrin and the other salivary vitamin-binding proteins during different stimulatory states has been neglected. Indeed, there have been reported cases of haptocorrin deficiency with no associated hypovitaminosis B_{12}, further questioning the significance of haptocorrin to vitamin B_{12} assimilation (226). Of the three pairs of major salivary glands, the rate of parotid gland secretion is the most up regulated when stimulated by mastication (223). Consequently; it might be expected that parotid glands would synthesis the majority of vitamin-binding proteins if they exert a function in vitamin assimilation. The major salivary glands are preferential innervated by different nerves

and activated, to varying extents, by different stimuli. For example, olfaction only stimulates submandibular and sublingual glands. Whereas food (by taste and mastication) upregulates parotid glands more than submandibular or sublingual, the parotid glands contribute to 58% of salivary volume with mechanical stimulation compared to 21%, with no stimulation, or 45% with citric acid stimulation (227).

4.5.1 Salivary vitamin D-binding protein

Vitamin D-binding protein is highly abundant in the circulation, predominantly hepatically expressed, with a low degree of transcription occurring in extra-hepatic tissues such as kidney, testis and abdominal adipose (32). The data presented in Chapter 3 demonstrated salivary DBP concentration was 1000-fold lower than that of serum, DBP concentrations across the two biofluids shared a positive relationship. DBP was initially identified as a salivary constituent in the 1980s, being recognised in parotid secretions by crossed immunoelectrophoretic mapping (228). The work presented in this chapter supports the work of Krayer *et al.* who quantified DBP to a range of ~1-100ng/mL in whole mouth and parotid saliva by ELISA (140). No clear change in whole mouth salivary DBP concentrations between rest and chew stimulated flow was found; however, DBP output did increase with stimulation. The parotid contribution however, (Figure 4-3) decreased in concentration with stimulation but maintained its output, thus suggesting DBP does not enter the parotid secretion as a typical acinar product. Instead DBP in parotid secretions may be secreted by ductal cells, which would allow DBP concentration to gradually increase at resting flow but would be diluted with stimulation.

The greatest source of DBP in whole mouth saliva is non-parotid in origin as demonstrated in Figure 4-3. Data presented in Figure 3-3 represents a positive relationship between salivary and serum DBP, Figure 4-3 in this chapter demonstrated the majority of WMS DBP was not parotid in origin. The WMS(NP) samples in this study would contain secretions from the sublingual and submandibular glands, minor gland saliva, and contaminants from the gingival crevicular fluid (GCF). GCF is a serum exudate which pools in the gingival sulcus. The periodontium is a highly vascularised tissue allowing the migration of circulatory molecules and cells out of blood vessels into the tissue (229). Being a serum exudate, GCF greatly reflect the composition of serum and in a healthy state contains an even greater concentration of DBP than that of serum (141). The method of stimulation in this study may be responsible for the large increase in WMS(NP) DBP between rest and stimulated demonstrated in Figure 4-2. Mastication increases salivary concentration of epithelial and microbial cell output and could increase the flow of GCF contamination, the high DBP concentration in GCF, could therefore be

responsible for the observed increases with mastication (230). Inflammation of the periodontium can result in a faster rate of exudation from the circulation (231, 232), and therefore GCF may have a higher concentration of serum components, this may be responsible for the dichotomous inter-personal trends observed in Figure 4-2 (a). In addition to its role in vitamin D transport, vitamin D-binding protein has been demonstrated to have immunomodulatory functions, enhancing the chemotactic activity of the complement pathway components C5a and $C5a^{desArg}$ (40), as well as scavenging actin-monomers, preventing filamentation which may occlude the vasculature (233). An abundance of DBP in the GCF may have a protective role for the periodontium.

4.5.2 Salivary haptocorrin

Figure 4-5 (a) demonstrates whole mouth salivary haptocorrin consistently decreased in concentration with mastication stimulation, Hc output did however, increase with stimulation. This appears to be consistent for parotid and non-parotid secretions, implying expression by salivary gland ductal cells. Haptocorrin has been detected by others in saliva, and immunohistochemically in both ductal and acinar mucous cells of salivary glands (234-236). Since the parotid glands do not contain mucus acini, they would produce less haptocorrin than the submandibular and sublingual glands. This may suggest that haptocorrin has additional roles to the assimilation of vitamin B_{12} because of the continual secretion of haptocorrin without stimulation.

Haptocorrin is not responsible for vitamin B_{12} absorption across the gastro-intestinal epithelium; indeed, a failure to proteolytically dissociate Hc from vitamin B_{12} in the duodenum can lead to malabsorption (81, 226). Instead an homologous binding protein termed intrinsic factor (IF), binds the released vitamin, and facilitates receptor mediated endocytosis in the terminal ileum (79). Unlike the vitamin B_{12}-binding site of IF, that of Hc has poor discrimination between the physiologically active and inactive analogues in humans (94). Hc has been identified in a number of exocrine secretions in addition to saliva such as tears, milk, nasal secretion, and seminal plasma (237-239). Furthermore, haptocorrin is a component of neutrophil secretory granules along with the iron sequestering protein, lactoferrin (226). This conserved secretion onto numerous mucosal surfaces and the non-selective ability to scavenge vitamin B_{12} analogues may suggest an antimicrobial function.

4.5.3 Salivary retinol-binding protein

Retinol-binding protein has been detected in parotid secretions by others (240). Figure 4-7 and Figure 4-8 indicate the output of RBP increased with mastication stimulation, the

parotid saliva also contained RBP which is complimentary to the aforementioned study. RBP is an abundant circulatory protein predominantly expressed by hepatocytes, RBP transcripts however, have also been detected in adipose tissue, and to a lesser extent renal tissue (125). Structurally RBP exhibits a single binding site for all-*trans* retinol, and is complexed to transthyretin in the circulation which increases the collective molecular weight, reducing the degree of glomerular filtration (121). Being a lipid soluble vitamin, the orientation of the hydrophobic domains of the molecule are shrouded within a beta-barrel hydrophobic pocket, which increases the solubility of the complex (240). As with DBP, the trend in salivary RBP concentration between resting and mastication stimulated flow varied between participants (Figure 4-7 (a)). This variance may be a consequence of gingival inflammation, and increased contamination from the circulation. Previous studies have detected RBP in mouse salivary glands; however, localisation within the tissue was not determined (241). The circulatory concentration of RBP demonstrated in Figure 3-9 correlates well with vitamin A status; indeed, there is literature which shows hepatocytes expression of RBP is driven by retinol status (242). Figure 3-9 shows a lack of correlation between salivary RBP and retinol which questions the role of retinol in regulating the expression of RBP in extra-hepatic tissues.

4.5.4 Summary

The work presented in this chapter indicated that DBP, Hc, and RBP all increased in whole mouth saliva output with mastication stimulation suggesting they are secreted by salivary glands, although which cell type may differ between the three proteins and glands. These data demonstrate that there would be large amounts of the three proteins during digestion, furthermore high concentrations even at resting salivary flow, may indicate a role for the proteins in maintaining oral homeostasis which will be explored in subsequent chapters.

Chapter 5 The influence of periodontal disease on salivary vitamin-binding proteins and vitamin status, a longitudinal cohort study

5.1 Rationale

Periodontal disease is a chronic inflammatory disorder affecting the supportive tissues of the dentition (periodontium). The aetiology of periodontal disease is considered to be a disruption of homeostasis in the tissues adjacent to the multispecies biofilms (plaque) which form on the enamel pellicle (168). There remains a lack of clarity as to the initial cause of periodontal disease, be it host, or microbial. A number of bacterial species demonstrate a strong association with the disease and have subsequently been termed the “red complex’ (187, 243). Attributes of these species have been demonstrated to dysregulate the typical immune response and maintenance of these tissues. One such species is the Gram negative, anaerobic pathobiont *Porphyromonas gingivalis*, which is a common commensal species in plaque, and is frequently enriched in plaque bordering sites of gingival inflammation (244). *P. gingivalis* is considered a keystone species which can promote a dysbiotic shift in the microbial community composition (185, 188). A well-defined virulence factor of *P. gingivalis* are a group promiscuous trypsin-like cysteine proteases termed gingipains (189).

A significant, and current literature has demonstrated strong associations between periodontal disease and a range of systemic diseases and health concerns, including diabetes and cardiovascular disease (173, 245, 246). A number of studies have highlighted the association between micronutrient deficiencies with periodontal disease, the most substantiated being hypovitaminosis D, but a number of studies have further demonstrated relationships with hypovitaminoses B_{12} and A (107). The discovery of the vitamin D-receptor has greatly broadened understanding of the importance of vitamin D for maintaining health, and particularly its role in modulating inflammatory and immune responses (64). Retrospective epidemiological studies using large datasets such as NHANES have demonstrated inverse associations between a number of vitamins and periodontal disease severity (247). A study by Zong *et al.* (2016) reported an inverse association between vitamin B_{12} status, and clinical markers of periodontal disease progression (107)

5.2 Aims

To compare concentrations of salivary vitamin-binding proteins, and serum vitamin levels in a periodontal disease cohort, before and after treatment, to understand the influence of periodontal disease on salivary vitamin-binding proteins and ultimately vitamin status.

5.3 Method

5.3.1 Ethical approval

This study used a pre-collected sample set kindly made available for analysis by Professor F. Hughes, Centre for Host-Microbiome Interactions, Faculty of Dentistry, Oral and Craniofacial Sciences. Ethical approval was obtained by Professor F. Hughes, from the proportionate review sub-committee of the HR, NRS Committee North East – Newcastle, and North Tyneside 2. Rec reference 12/NE/0122.

5.3.2 Study design

Participants received an initial periodontal examination at baseline, followed by a standardised course of nonsurgical periodontal treatment consisting of oral hygiene instruction and full mouth subgingival debridement with local anaesthesia. Samples were collected at the baseline visit and at follow up visit 10 weeks following completion of treatment. All treatment was carried out by the same operator who was an experienced trained periodontist (Dr Belkais Abushaia Karim).

5.3.3 Cohort disease characterisation

All participants were diagnosed with moderate to severe periodontitis according to AAP / CDC case definition criteria (i.e. Patients with two or more interproximal sites with clinical attachment loss of ≥ 4mm occurring at two or more different teeth *or* two or more interproximal sites with a probing depth of ≥5mm, not on the same tooth) (248). Response to treatment was assessed according to the percentage of deep pockets that showed no response to treatment as defined by pocket depth reduction. Poorly-responding patients were defined as having more that 50% of non-responding deep sites (249).

5.3.4 Sample analysis

Details of specific analyses can be found in greater detail in Chapter 2 Materials and methods. In brief, saliva was analysed by ELISA, to quantify vitamin D-binding protein, haptocorrin, and retinol-binding protein. Salivary proteins were separated by SDS-PAGE electrophoresis, before immunoblotting for vitamin D-binding protein and haptocorrin to visualise any obvious indications of degradation. 25-OH-D and total-vitamin B_{12} were

quantified in serum by chemiluminescent microparticle immunoassay (CMIA), as markers of vitamin D and B_{12} status respectively. Retinol was quantified by HPLC as a marker of vitamin A status.

Due to limited sample volume it was not always possible to conduct all assays on all sample types, full details of sample size per assay can be found in Appendix 13 and Appendix 14.

5.3.5 Statistical analysis

Distribution of all data sets was determined with D'Agostino and Pearson normality test. Paired data were analysed with paired t-test, or a non-parametric equivalent (noted in results where appropriate). Multiple group analysis was undertaken with ANOVA, and multiple comparison tests, specific tests are noted in figure captions.

5.4 Results

Table 5-1 Periodontal disease cohort summary

	Aggregate	**Gender**		**Smoking status**		
		Female	**Male**	**Current smoker**	**Previous smoker**	**Never smoked**
		Mean±SEM		**Mean±SEM**		
n	30	17	13	8	11	11
Age	44.3±1.62	43.7±1.76	45.1±3.03	41.13±3.19	47.82±2.48	43.09±2.73

Raw data can be found in Appendix 12.

5.4.1 Vitamin D and vitamin D-binding protein

Figure 5-1 is a series of immunoblots of longitudinally paired saliva samples from the periodontal disease cohort, before and after treatment, probed for vitamin D-binding protein. All samples indicate a band at the correct molecular weight. There is no clear indication of degradation, some differences are evident between paired samples before and after treatment, for example the band intensity is greater in 12A than 12B, and 23B than 23B.

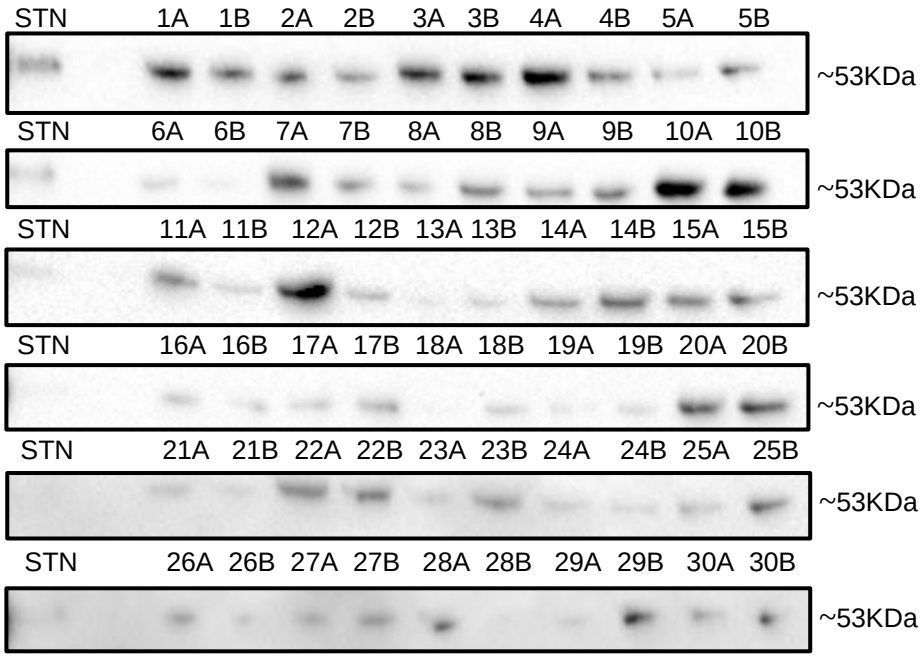

Figure 5-1 Immunoblot of salivary vitamin D-binding protein in a periodontal disease cohort.
(1-30) prior to treatment (A), and post treatment (B). Blot probed for vitamin D-binding protein. STN – vitamin D-binding protein standard.

Salivary DBP concentration decreased in 73% of patients after treatment, which was statistically significant when analysed by non-parametric, paired statistical analysis (Figure 5-2 (a)) (raw data is presented in Appendix 14). 63% of patients had a decrease in serum 25-OH-D concentration after treatment, however this was not significant (Figure 5-2 (b)) (raw data is presented in Appendix 13).

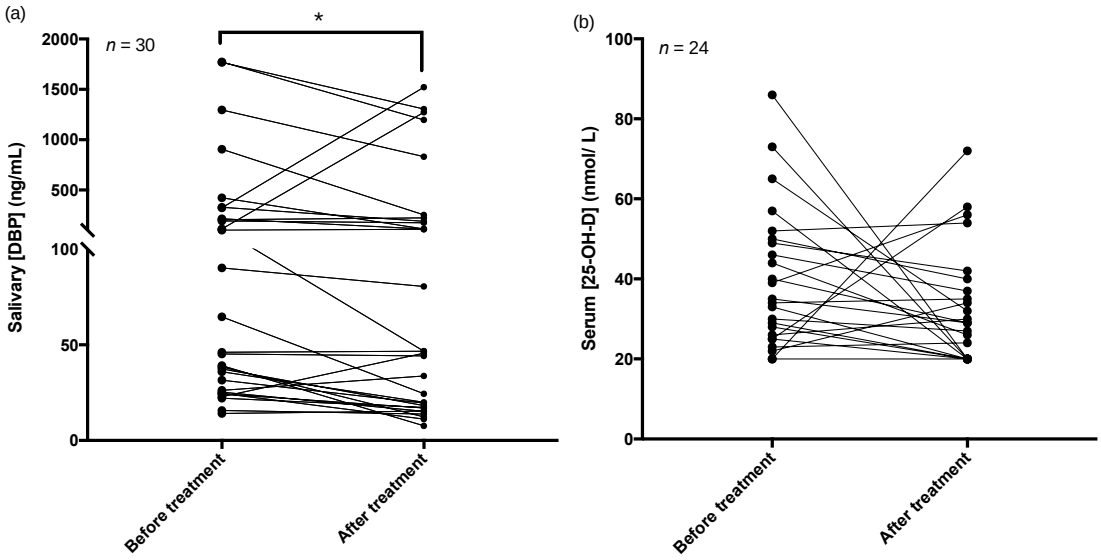

Figure 5-2 Influence of periodontal disease on salivary vitamin D-binding protein concentration, as quantified by ELISA, and circulatory 25-OH-D concentration, as quantified by CMIA

(a) Subject paired salivary vitamin D-binding protein concentration before and after treatment for periodontal disease, a significant decrease in DBP concentration is observed when analysed by Wilcoxon matched pairs signed rank test (∗ = $p < 0.05$).

(b) Subject paired circulatory 25-OH-D concentration before and after treatment for periodontal disease, no significant difference was observed.

Compared to the periodontally healthy cohort (Figure 3-3 (a)), DBP concentration was significantly higher in the disease cohort post treatment, assessed by non-parametric ANOVA, and multiple comparisons test. No difference was found between the healthy and disease cohort at either timepoint for serum 25-OH-D concentration (Figure 5-3 (b)). The high incidence of 25-OH-D insufficiency (50-30nmol/L) was high in both cohorts 36% in the healthy cohort, and 33% in the periodontal disease cohort decreasing to 25% after treatment. Deficiency of 25-OH-D (<30nmol/L) was also high in both cohorts with 26% of healthy participants deficient, and 45% of periodontal disease patients increasing to 58% post treatment (Figure 5-3b) (raw data Appendix 13).

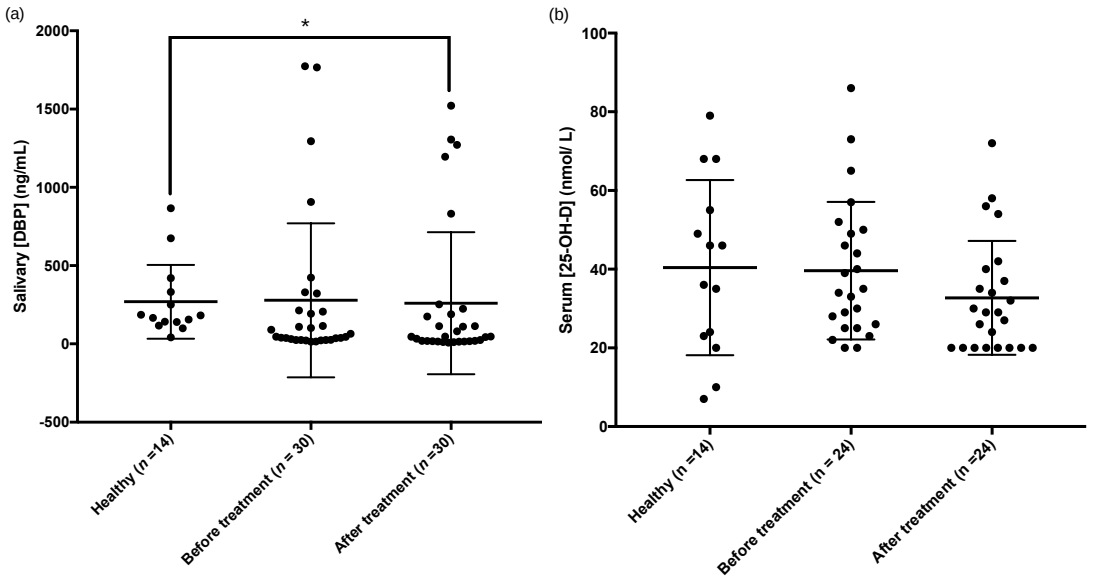

Figure 5-3 Comparing salivary vitamin D-binding protein and circulatory 25-OH-D concentrations between health and disease

Individual measurements and group means are presented, error bars indicate standard deviation

(a) Salivary vitamin D-binding protein concentration in a periodontally healthy cohort, compared to a periodontal disease cohort before and after treatment. Significant difference was seen between the health and after treatment cohorts when assessed by Kruskal-Wallis test, and Dunn's multiple comparisons test (∗ = $p < 0.05$).

(b) Circulatory 25-OH-D concentration in a periodontally healthy cohort, compared to a periodontal disease cohort before and after treatment. No significant difference was observed.

No statistical differences in salivary DBP (Figure 5-4 (a)) and serum 25-OH-D Figure 5-4 (b)) concentrations in patient groups the either responded, or failed to respond to treatment, as defined in 5.3.3.

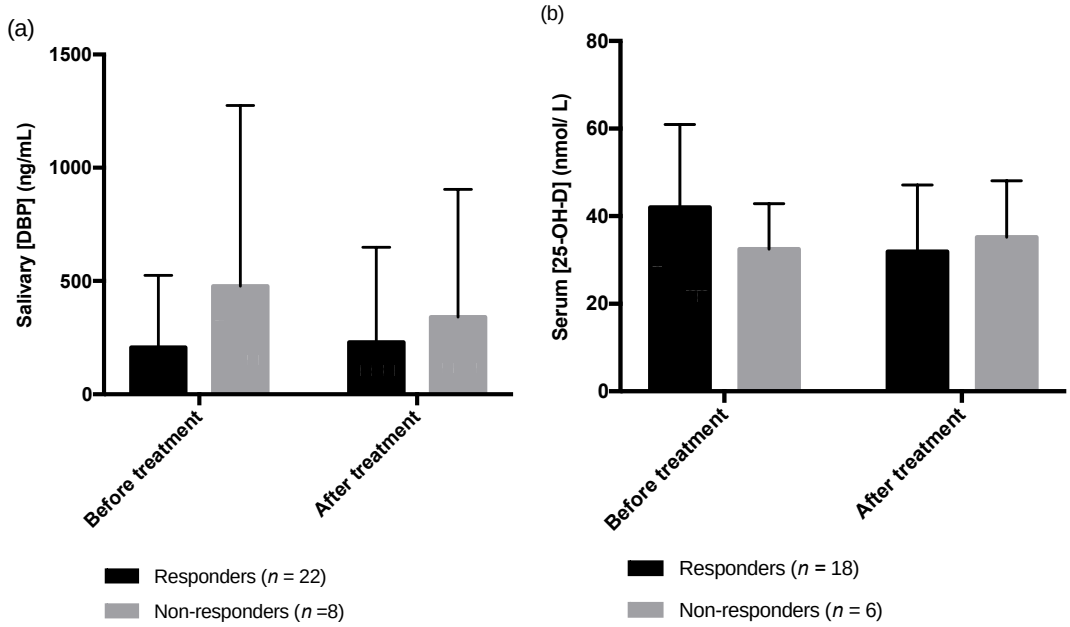

Figure 5-4 Differences in salivary vitamin D-binding protein, and serum 25-OH-D concentrations in response to periodontal disease treatment

(a) Salivary DBP concentration before and after treatment for periodontal disease, divided by response to treatment

(b) Serum 25-OH-D concentration before and after treatment for periodontal disease, divided by response to treatment

5.4.2 Vitamin B12 and haptocorrin

Figure 5-5 is a series of immunoblots probed for haptocorrin. Longitudinally paired before (A) and after (B) treatment saliva samples of 30 periodontal disease patients. Compared to the haptocorrin immunoblot presented in Figure 3-1(ii) from a cohort of periodontally healthy individuals, the bands presented in Figure 5-5 are more diffuse and less defined. There are multiple examples where the band intensity is greater in the post treatment samples such as 2A and B, 5A and 5, and 21A and B. A laddering banding pattern is demonstrated in 14A, these observed features may be a consequence of enzymatic cleavage of constituents of haptocorrin, be it proteolytic or glycolytic.

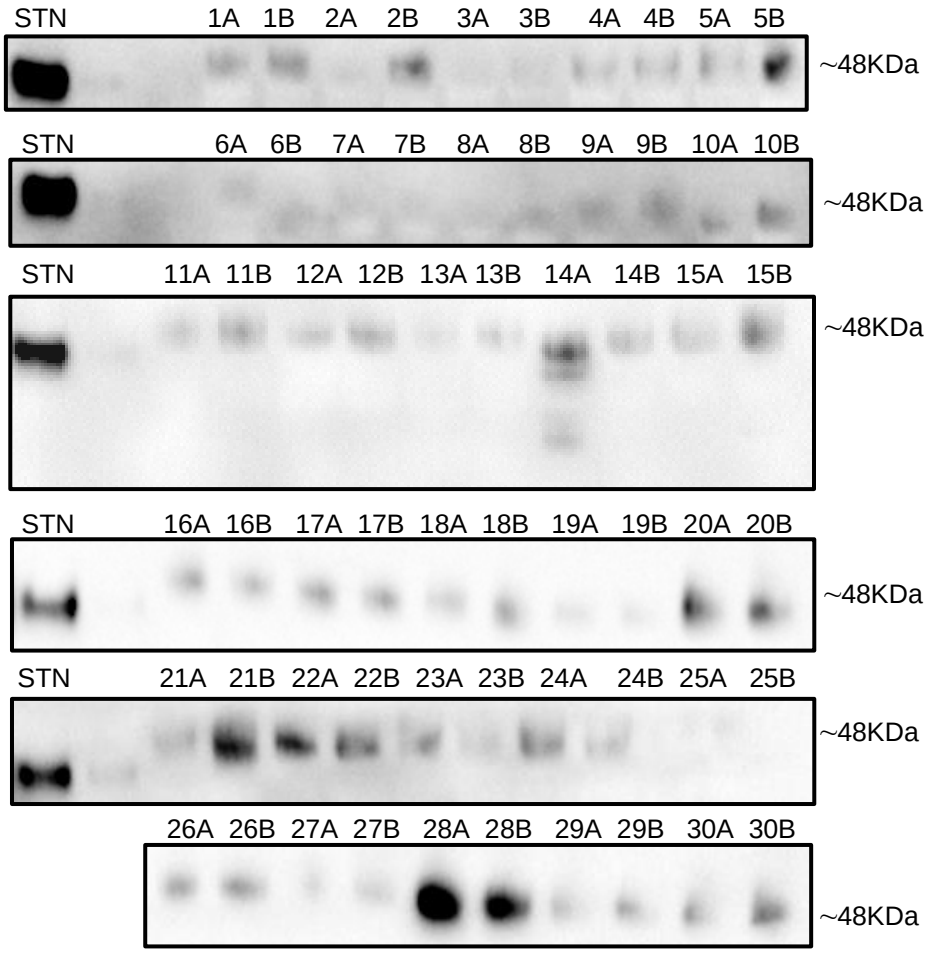

Figure 5-5 Immunoblot of salivary haptocorrin in a periodontal disease cohort (1-30) prior to treatment (A), and post treatment (B). Blot probed for haptocorrin. STN – haptocorrin standard.

The concentration of salivary haptocorrin was greater in 77% of periodontal disease patients after treatment (Figure 5-6 (a)) which was statistically significant. Non-parametric distribution was determined by D'Agostino & Pearson normality test, and subsequently the data were assessed by Wilcoxon matched-pairs signed rank test which demonstrated significant difference between the two groups. Total-B_{12} was quantified in serum and demonstrated no clear trend between sample points (Figure 5-6 (b)) with 45%

of patients having a greater concentration after treatment. Distribution of the data was determined by the aforementioned normality test and the subsequent paired t-test indicated no significant difference before and after treatment.

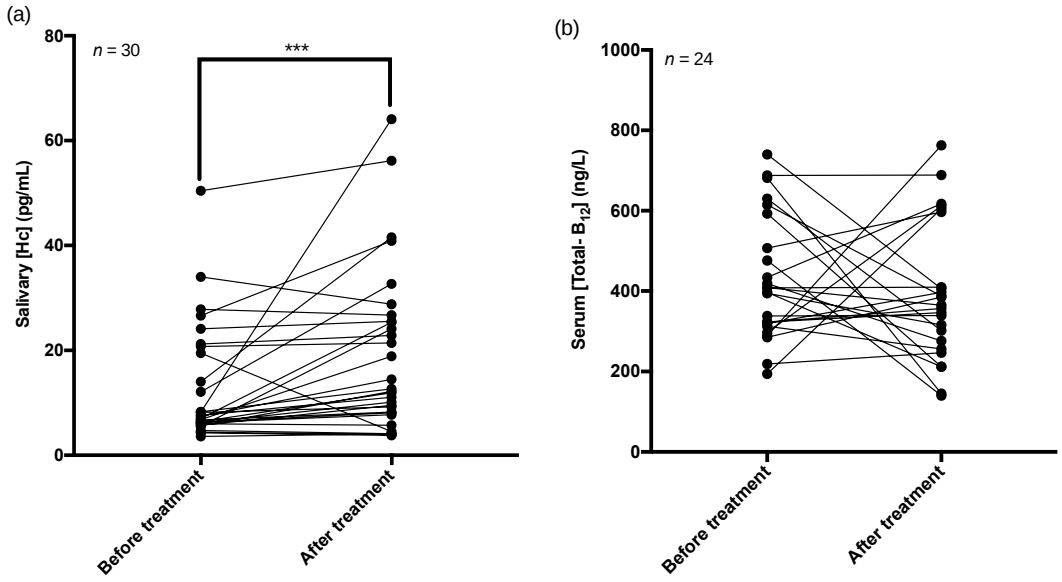

Figure 5-6 The influence of periodontal disease on salivary haptocorrin concentration, as quantified by ELISA, and circulatory total-vitamin B_{12} concentration, as quantified by CMIA

(a) Subject paired salivary haptocorrin concentration before and after treatment for periodontal disease, a significantly higher concentration was observed after treatment (*** = $p < 0.001$) when analysed by Wilcoxon matched-pairs signed rank test.

(b) Subject paired circulatory total-B_{12} concentration before and after treatment for periodontal disease, no significant difference was observed.

Salivary haptocorrin concentration was greater in the periodontal disease cohort when compared to controls, both before and after treatment, less significantly so in the latter (Figure 5-7 (a)). Conversely, circulatory total-B_{12} was significantly lower in the healthy cohort compared to the disease cohort both before and after treatment (Figure 5-7 (b))

A significantly higher salivary haptocorrin concentration was seen in the periodontal disease patient which responded to treatment, no other significant differences were found for non-responsive patients, or for serum total-B_{12} concentration for any cohort (Figure 5-8).

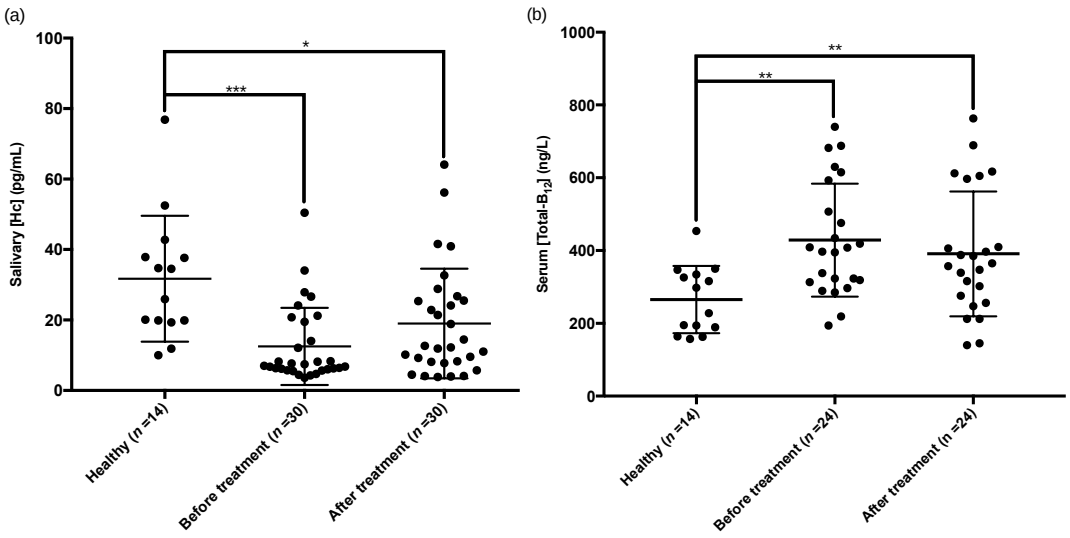

Figure 5-7 Comparing salivary haptocorrin and total-B_{12} concentrations between health and disease

Individual measurements and group means are presented, error bars indicate standard deviation

(a) Salivary haptocorrin concentration in a periodontally healthy cohort, compared to a periodontal disease cohort before and after treatment. Significant difference was observed between the healthy cohort, and before treatment (∗∗∗ = p <0.001), and after treatment (∗ = p <0.05), when analysed by Kruskal-Wallis test, and Dunn's multiple comparisons test.

(b) Circulatory total vitamin B_{12} concentration demonstrates significant deference between the healthy cohort and the periodontal disease cohort before and after treatment (∗ = p <0.05), when analysed by one-way ANOVA and Tukey's multiple comparisons test

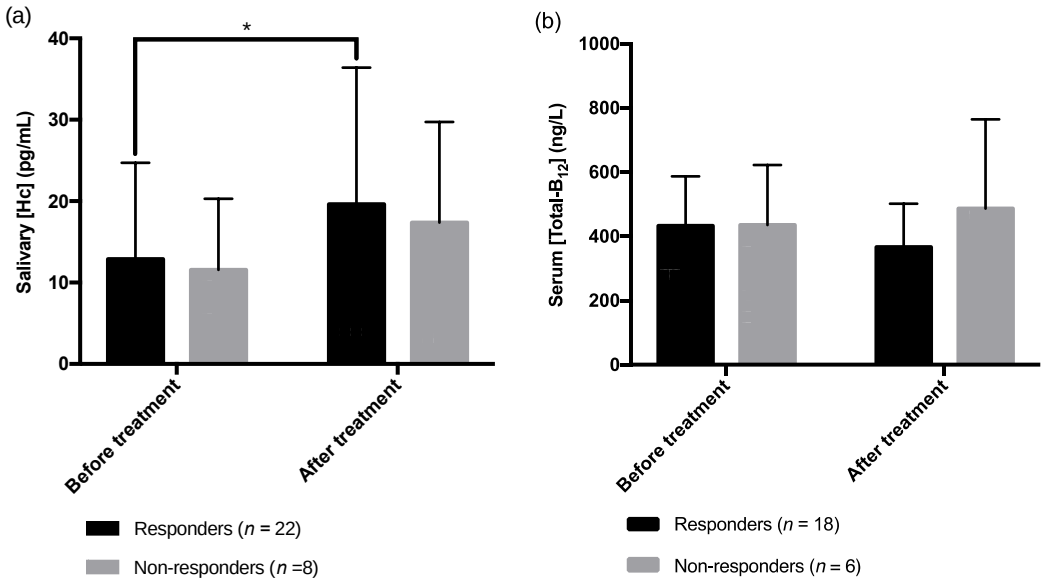

Figure 5-8 Differences in salivary haptocorrin, and serum Total-B_{12} concentrations in response to periodontal disease treatment

(c) Salivary Hc concentration before and after treatment for periodontal disease, divided by response to treatment (∗ = p <0.05).

(d) Serum total-B_{12} concentration before and after treatment for periodontal disease, divided by response to treatment

5.4.3 Vitamin A and retinol-binding protein

No trend was demonstrated in salivary retinol-binding protein concentration between sample points (Figure 5-9 (a)), when quantified by ELISA. The data presented a non-parametric distribution and therefore was assessed with Wilcoxon matched-pairs signed rank test, from which no significant difference was detected. Serum retinol concentration did not significantly change in the periodontal disease cohort with treatment (Figure 5-9 (b)).

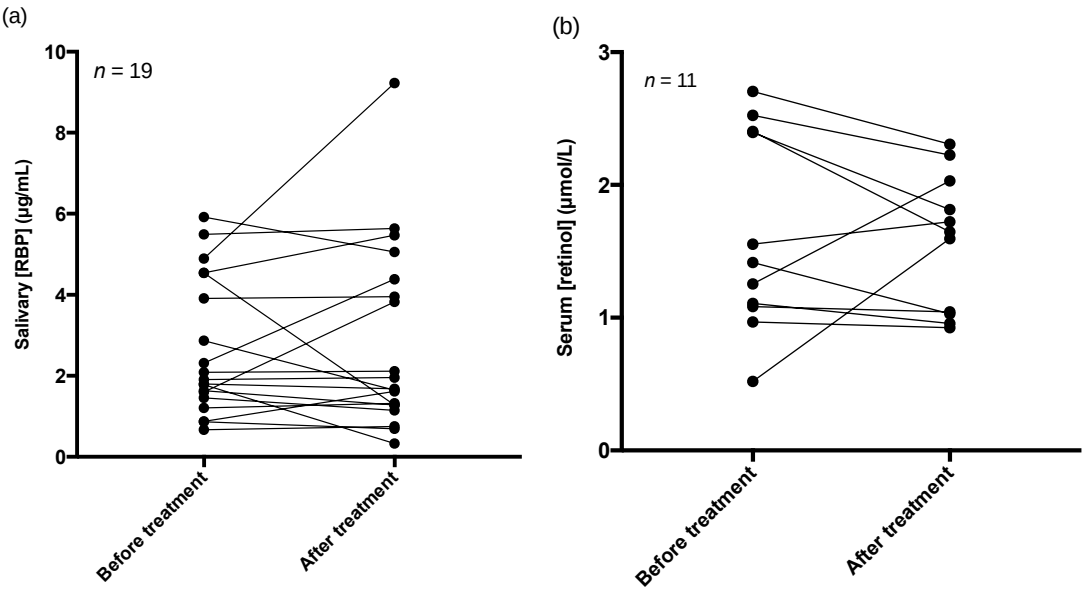

Figure 5-9 The influence of periodontal disease on salivary retinol-binding protein concentration, as quantified by ELISA, and circulatory retinol concentration, as quantified by HPLC

(a) Subject paired salivary retinol-binding protein concentration before and after treatment for periodontal disease, no significant difference between groups
(b) Subject paired circulatory retinol concentration before and after treatment for periodontal disease, no significant difference was observed.

Figure 5-10 (a) demonstrates no statistical change was observed in salivary retinol-binding protein concertation between health and disease, when assessed by Kruskal-Wallis, and Dunn's multiple comparisons tests. Interestingly serum retinol concentration was lower in periodontal disease patients before treatment compared to controls Figure 5-10 (a). Analysis was undertaken with one-way ANOVA, and Tukey's multiple comparisons tests.

No significant differences were found between treatment responders and non-responders for either salivary RBP or serum retinol concentrations (Figure 5-11).

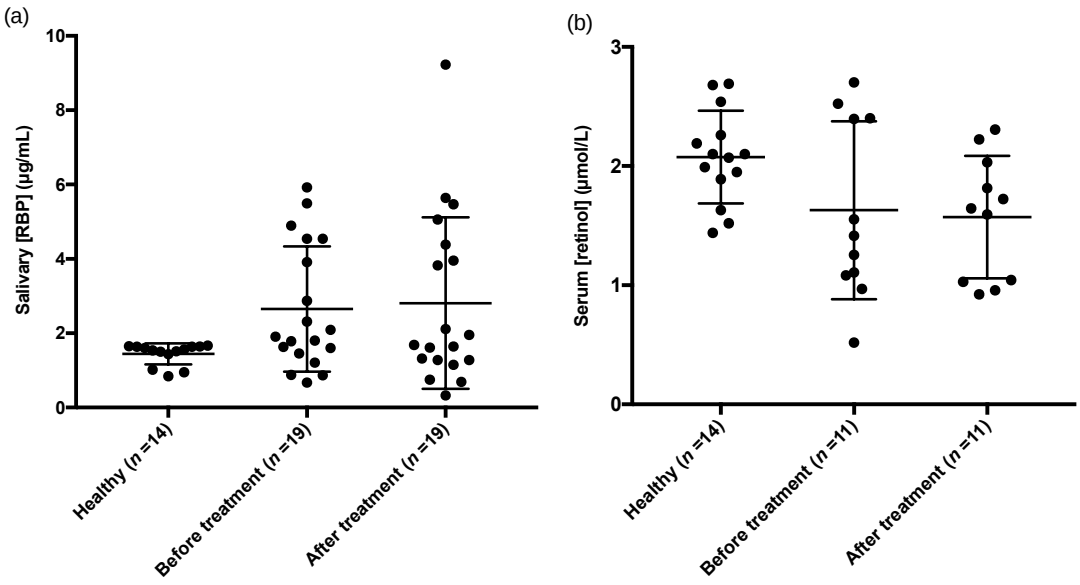

Figure 5-10 Comparing salivary retinol-binding protein and retinol concentrations between health and disease

Individual measurements and group means are presented, error bars indicate standard deviation

(a) Salivary retinol-binding protein concentration in a periodontally healthy cohort, compared to a periodontal disease cohort before and after treatment.

(b) Circulatory retinol concentration in the healthy cohort and the periodontal disease cohort before and after treatment. Data presented a normal distribution, one-way ANOVA was undertaken, and did not find significant differences between groups

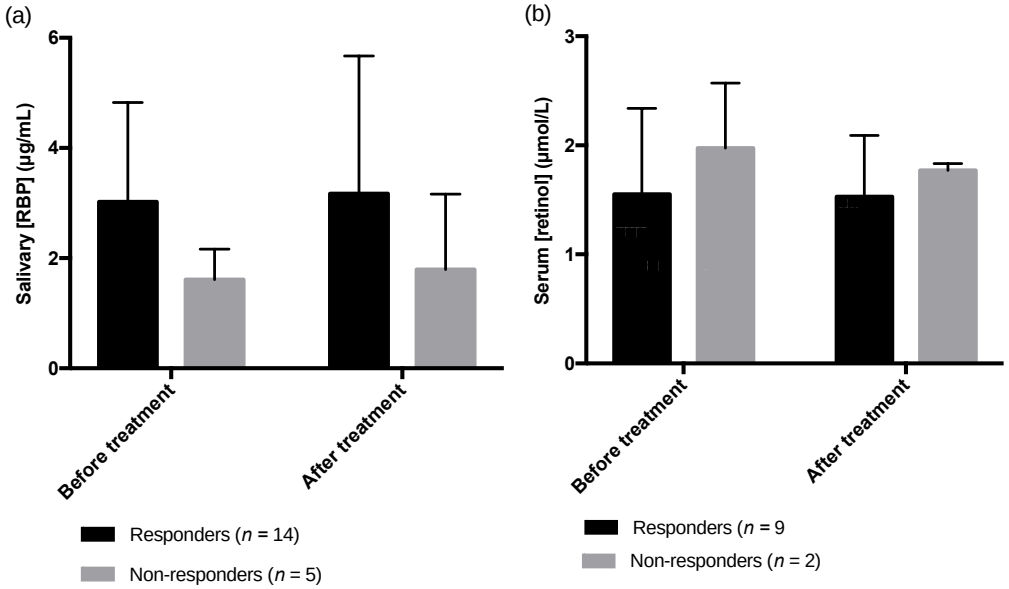

Figure 5-11 Differences in salivary retinol-binding protein, and serum retinol concentrations in response to periodontal disease treatment

(a) Salivary RBP concentration before and after treatment for periodontal disease, divided by response to treatment

(b) Serum retinol concentration before and after treatment for periodontal disease, divided by response to treatment

5.5 Discussion

5.5.1 Summary

The data presented in this chapter demonstrated that periodontal disease had apposing effects on the salivary concentrations of vitamin D-binding protein and haptocorrin, revealing a significant increase and decrease, respectively. These changes in salivary vitamin-binding protein concentration do not correlate positively with changes in systemic markers of vitamin status, and therefore questions the significance of salivary vitamin-binding proteins in assimilation of their vitamin ligands.

5.5.2 Vitamin D and vitamin D-binding protein

The results in this chapter suggest periodontal treatment had no influence on 25-OH-D status in this cohort, both before or after treatment, or indeed when compared to a periodontally healthy cohort.

The literature surrounding sub-optimal vitamin D status and periodontal disease remains inconclusive. A number of large epidemiological analyses including the use of the National Health and Nutrition Examination Survey 3 (NHANES 3) generated in the USA, have identified inverse associations between 25-OH-D status and gingival inflammation (56). Vitamin D status at the point of periodontal surgery has been demonstrated to affect surgical outcomes, and indeed supplementation with calcium and vitamin D has been demonstrated to improve periodontal health in those on periodontal maintenance therapy (68, 250). Mechanistically the critical importance of vitamin D for the regulation of calcium homeostasis and subsequently bone mineral density has long been known, with clinical deficiency presenting systemically as osteopenia and osteoporosis (49). In terms of oral health, numerous studies have highlighted the relationship between systemic and oral bone mineral density, in particular mandibular and alveolar bone density (251-254). Vitamin D acting via the nuclear vitamin D-receptor: VDR, has been shown to attenuate osteoclastogenesis and subsequently reduce alveolar bone resorption (255). The literature indicates the protective effect of vitamin D may be irrespective of bone mineral density (55). Although 25-OH-D is a commonly employed and robust indicator of vitamin D status, it is activated by a second hydroxylation step undertaken by 1 α-hydroxylase which is expressed in gingival tissue. The subsequent 1,25-dihydroxyvitamin D has been demonstrated to have immune and barrier functioning attributes, and therefore may offer localised immune-protective effects (256, 257). Measuring these two vitamin D metabolites in gingival crevicular fluid may offer a better indication to the protective role of vitamin D in gingival health.

The findings of this chapter indicated a higher whole mouth salivary concentration of vitamin D-binding protein (DBP) before treatment. This finding complements a study by Krayer *et al*, which quantified DBP by ELISA in whole mouth, and parotid saliva, and noted a greater whole mouth DBP concentration with periodontal disease (140). With DBP being highly abundant in both the circulation and GCF, the increase in salivary DBP could be a consequence of gingival inflammation and increased contamination from these fluids, which has been demonstrated previously for other circulatory proteins (258). Interestingly, a study by Zhang *et al,* observed higher plasma concentration of DBP in patients with generalised aggressive periodontitis (199). The trend (positive, or negative) in salivary concentrations of DBP between sample points varied between patients, although clinically the group were homogenous, the degree of blood contamination at a given point may vary between patients and therefore influence the result.

Vitamin D-binding protein is multifunctional. In addition to its vitamin D-binding domain, it also possesses a G-actin-binding site and acts to sequester actin-monomers preventing unregulated filamentous actin formation (F-actin) (259, 260). G-actin can be released into the Interstitium and enter the circulation with tissue injury, and therefore, DBP may have a protective role in the periodontium during inflammation by sequestering free actin (261). Indeed, the aforementioned Krayer *et al,* study identified the majority of salivary DBP was complexed with G-actin in periodontal disease (140). Salivary DBP concentration was collectively lower in those patients which responded to treatment. Although this finding was not significant, a greater sample size might be useful to demonstrate a difference.

This section of work complements the findings presented in chapter 4.4.2 which suggested a significant contribution of salivary DBP was from the gingival crevicular fluid, and therefore could be a potential quantitative biomarker for periodontal inflammation, or an indicator of response to treatment. The literature highlights the potential importance of vitamin D-binding protein in supporting periodontal health.

5.5.3 Vitamin B_{12} and haptocorrin

A paper published by Zong *et al,* in 2016 demonstrated an inverse association between serum vitamin B_{12} status and periodontal disease progression and tooth loss in a cohort of >1600 patients with a multivariant regression model. Probing pocket depth (PD) and clinical attachment loss (CAL) were used as markers of periodontal disease severity (107). The authors did not offer a suggestion on mechanism. The study also found no association between smoking and vitamin B_{12} status which is complimentary to research

in this chapter. The findings presented here demonstrated no clear difference in total-B_{12} before or after treatment for periodontal disease. However, the periodontal disease cohort presented higher serum levels of total-B_{12} than the periodontally healthy cohort, which may be a consequence of dietary differences between the two groups. The sensitivity of vitamin B_{12} biomarkers is an issue which has affected a number of studies and has led to a search for more reliable alternatives (262). A recent, more suitable biomarker may be holotranscobalamin, which quantifies the fraction of serum B_{12} bound to the circulatory binding protein transcobalamin-II (TCN-II), this is the portion of B_{12} available to cells, and has proven to be more sensitive to acute changes in vitamin B_{12} status (263). Dissimilar to the other water-soluble vitamins, vitamin B_{12} is extensively stored in hepatic parenchyma, and therefore inadequate intake can take a long time to be detected, both biochemically and clinically (264).

In this present study, salivary haptocorrin was observed at a lower concentration in the periodontal disease cohort prior to treatment, compared to post treatment. Both these sample time points demonstrated lower haptocorrin concentration compared to a periodontally healthy cohort. Thus, suggesting periodontal disease may be a driver of reduced salivary haptocorrin concentration, possibly as a consequence of proteolytic degradation, which is supported by protein band laddering on immunoblots (Figure 5-5). A significant increase in post-treatment salivary haptocorrin concentration was observed in patients that responded to treatment. The reasoning for disparity in the cohort, with regard to treatment outcome is unclear. Salivary haptocorrin is often referred to in the literature as an important intermediary protein in the vitamin B_{12} assimilation pathway (79, 214). Although haptocorrin was detected in all samples, there was significantly lower concentrations prior to treatment, which had no clear association with vitamin B_{12} status. This could question the significance of salivary haptocorrin to vitamin B_{12} assimilation or could suggest there is an excess of salivary haptocorrin and therefore some degradation may not influence assimilation. The gastric mucosa is also a potential source of haptocorrin and therefore may add redundancy to salivary haptocorrin (234).

5.5.4 Vitamin A and retinol-binding protein

Nutritional studies that have focused on periodontal disease have suggested that dietary supplementation with antioxidant vitamins including vitamin A, and the previtamin-A carotenoids are associated with periodontal disease severity and supplementation can support positive treatment outcomes (137, 265). Data presented in this study indicated lower circulatory retinol concentration in disease compared to healthy controls, although

this was not significant, no difference was observed between controls and disease cohort post treatment; the antioxidant properties of vitamin A may contribute to this.

No change in salivary retinol-binding protein (RBP) was observed with treatment for periodontal disease, or between control and disease cohorts. The range of salivary RBP concentration was greater in the disease cohort compared to control. One study identified elevated serum RBP concentration in a periodontal disease cohort, which decreased three months after non-surgical treatment (266). RBP in gingival crevicular fluid has also been suggested as a marker for periodontal disease and obesity, demonstrating increases in concentration in GCF and serum (267).

5.5.5 Study limitations

Due to limited volume of samples available not all assays were complete for all subjects and therefore sample sizes vary across tests. The control cohort used in this study were generally 10 years younger than the periodontal disease cohort, work presented in Chapter 3 suggested age does not influence the variables measured in this study, apart from salivary RBP concentration. The data may also be affected by a lack of flow rate data for the periodontal disease cohort, and not having longer follow-up samples. A larger sample size might be required to truly assess differences in salivary RBP between health and disease.

Chapter 6 Interactions between the pathobiont; *P. gingivalis*, salivary haptocorrin, and vitamin B_{12}

6.1 Rationale

Work presented in Chapter 5 demonstrated a lower concentration of salivary haptocorrin in a periodontal disease cohort prior to treatment, compared to a ten-week post treatment follow-up. Both sample points (before and after treatment) demonstrated lower salivary haptocorrin concentrations compared to a periodontally healthy cohort. Immunoblot data indicated the reduced concentrations may be a consequence of proteolytic activity, with observed reduced band intensity and band laddering.

Porphyromonas gingivalis is a common commensal constituent of the oral microbiome with a strong association to periodontal disease. Although *P. gingivalis* has been isolated from numerous sites in the oral cavity, it is particularly enriched in plaque adjacent to inflamed sites (268). Key virulence factors of *P. gingivalis,* responsible for destruction of the dental supportive tissues and dysregulation of the immune response in the periodontium are a group of cysteine proteases termed 'gingipains' (189). Gingipains include two arginine- specific proteases RgpA and RgpB, and a lysine- specific gingipain Kgp, which can be secreted or membrane associated (269). The gingipains have been demonstrated to be important attributes for *P. gingivalis* to thrive in the subgingival environment including colonisation, immune evasion, and nutrient acquisition (270).

It is becoming increasingly apparent that the nutritional requirement of vitamin B_{12} is not limited to higher kingdom organisms, numerous microbes lacking the complete machinery for *de novo* biosynthesis are also dependent on exogenous sources (271). Furthermore, the functional effects of vitamin B_{12} in microbes are far more diverse with the discovery of B_{12}-riboswitches, which are ligand dependent regulators of transcription (272). Therefore; a host factor which sequesters vitamin B_{12} may have an important innate immune function.

6.2 Aims

To investigate interactions between the keystone species *Porphyromonas gingivalis*, vitamin B_{12} and salivary haptocorrin.

6.3 Method

6.3.1 Culture maintenance

Porphyromonas gingivalis wild-type strain W50, and two isogenic Arg-, Lys-gingipain deletion knock-outs strains; E8 (RgpA/B) and K1A (Kgp) (kindly made available by Professor J. Naglik, Centre for Host-Microbiome Interactions) were maintained on Fastidious Anaerobic Agar (FAA) (Lab M, Heywood, U.K.), supplemented with 5% v/v defibrinated horse blood (E&O Laboratories Ltd, Bonnybridge, Scotland), at 37°C in a Whitley MG1000 anaerobic workstation (Don Whitley Scientific Ltd, Bingley, U.K.), with an atmosphere of 80% nitrogen, 10% carbon dioxide, and 10% hydrogen.

Streptococcus mutans NCTC 10449T (kindly made available by Steven Gilbert, Centre for Host-Microbiome Interactions) was maintained on Brain Heart Infusion agar (BHI) (Lab M, Heywood, U.K.) in the same anaerobic environment described for *P. gingivalis.*

6.3.2 Preparation of liquid cultures, and culture supernatants

Pre-reduced BHI broth supplemented with 5mg/mL haemin (Sigma-Aldrich, St. Louis, MI, U.S.A), was inoculated with *P. gingivalis* W50, E8 K1A, or *S. mutans* NCTC 10449T, and incubated in anaerobic conditions for 48 hours. The OD_{600} was measured and cultures normalised to an absorbance of 1 with media. Cultures were centrifuged at 13,000g for 10 minutes to pellet bacteria. Supernatant was divided into aliquots and stored at -80°C until use. Pellets were resuspended in the same volume of fresh media, prior to division into aliquots and storage at -80°C.

6.3.3 Investigating the interaction of oral pathobionts with haptocorrin

An aliquot of each culture supernatant was heated to 100°C for 15 minutes (referred to as pre-boiled supernatant). Recombinant haptocorrin (10ng/mL) (Abcam, Cambridge U.K.) was incubated with *P. gingivalis* W50 and *S.mutans* NCTC 10449T culture supernatants, or supernatant free media for 1 hour at either 37°C or 4°C (all combinations can be seen in Figure 6-2). Assay samples were then heated to 100°C for 2 minutes in the presence of NuPAGE LDS sample buffer (ThermoFisher, Waltham, MA, U.S.A.), and 1,4-Dithiothreitol (DTT) to halt the reaction. Samples were analysed by SDS-PAGE electrophoresis, western blot, and immunoprobed for haptocorrin (the full details of which can be found in Chapter 2).

6.3.4 Influence of titrating *P. gingivalis* supernatant on interaction with haptocorrin

Ten-fold dilution series were generated with *P. gingivalis* W50 culture supernatant and resuspended pellet, diluted with culture free media. This generated 11 dilutions ranging

from 100% - 0% v/v culture component. Recombinant haptocorrin was added to each dilution at a concentration 10ng/mL. The series was incubated at 37°C for 1 hour. Assay samples were then heated to 100°C for 2 minutes in the presence of NuPAGE LDS sample buffer and 1,4-Dithiothreitol (DTT) to halt the reaction. Samples were analysed by SDS-PAGE electrophoresis, western blot, and immunoprobed for haptocorrin (the full details of which can be found in Chapter 2).

6.3.5 *P. gingivalis* gingipain knock-out strains and interactions with haptocorrin

As described above, aliquots of culture supernatants from *P. gingivalis* wild-type parent strain W50, and two isogenic Arg-, Lys-gingipain knock-out strains; E8 and K1A were incubated for 1 hour at 37°C. Assay samples were then heated to 100°C for 2 minutes in the presence of NuPAGE LDS sample buffer and 1,4-Dithiothreitol (DTT) to halt the reaction. Samples were analysed by SDS-PAGE electrophoresis, western blot, and immunoprobed for haptocorrin (the full details of which can be found in Chapter 2).

6.3.6 Interaction between gingipains and native salivary haptocorrin

Ethical approval for human saliva collection was obtained from the Biomedical Sciences, Medicine, Dentistry and Natural and Mathematical Sciences subcommittee of the King's College London Research Ethics Committee (Reference: BDM/14/15-61). Whole mouth saliva was collected from two healthy individuals by expectoration into sterile universal tubes. Samples were divided into aliquots and centrifuged at 13,500rpm for 5 minutes at 45°C, salivary pellets were discarded. Each aliquot was divided into 4, each divided aliquot was then diluted 1:1 v/v with (a) culture free media, (b) W50 culture supernatant, (c) E8 culture supernatant or (d) K1A culture supernatant for 1 hour at 37°C. Assay samples were then heated to 100°C for 2 minutes in the presence of NuPAGE LDS sample buffer and 1,4-Dithiothreitol (DTT) to halt the reaction. Samples were analysed by SDS-PAGE electrophoresis, western blot, and immunoprobed for haptocorrin (the full details of which can be found in Chapter 2).

6.3.7 Vitamin B_{12} dependent *P. gingivalis* growth

P. gingivalis strain W50 culture was collected by loop and washed three times by suspended in phosphate-buffered saline (PBS), and centrifugation at 2000g for 3 minutes. The pellet was finally resuspended in pre-reduced defined medium which excluded vitamin B_{12} analogues and downstream nutrients of the vitamin B_{12} dependent pathways (See Appendix 16 for composition). Medium was supplemented with 5mg/mL Haemin, and 2.5mg/mL sodium-thioglycolate (Sigma-Aldrich, St. Louis, MI, U.S.A). Culture was normalised to by absorbance at 600nm to ~0.1. Cultures were loaded onto

a 96-well microtiter plate in triplicate and supplemented with either adenosylcobalamin (Sigma-Aldrich, St. Louis, MI, U.S.A) or methylcobalamin (Sigma-Aldrich, St. Louis, MI, U.S.A) to final concentrations of 1000pg/mL, 100pg/mL, or 0pg/mL (please refer to Figure 6-1). Additionally, 1000pg/mL concentration wells were supplemented with recombinant haptocorrin (final concentration of 1µg/mL). Plates were placed into an anaerobic chamber and sealed before loading onto iEMS 96-well microplate reader (MTX Lab Systems LLC Bradenton, FL, U.S.A.), incubated at 37°C, with gentle agitation, and recorded OD^{590} at 15-minute intervals, for a duration of 48 hours.

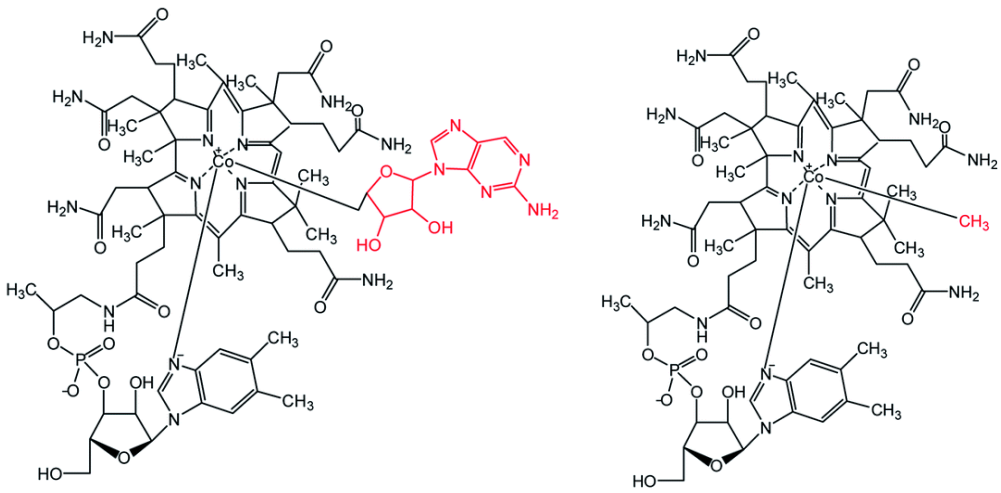

Figure 6-1 Chemical structures of vitamin B_{12} species
Adenosylcobalamin (left) and methylcobalamin (right).

6.4 Results

Figure 6-2 is a representative immunoblot probed for haptocorrin, and composite graph. Culture supernatant from (i), *P. gingivalis* strain W50 and (ii) *S. mutans* strain NCTC 10449T were incubated with recombinant haptocorrin under various conditions demonstrated in the figure table. Haptocorrin was not detectible via this method when incubated at 37°C for 1 hour with *P. gingivalis* culture supernatant, this was however, not the same for *S. mutans* (as demonstrated in lane 2). Haptocorrin was detectable by immunoblot if the supernatant was pre-boiled prior to incubation, or if the incubation was undertaken at 4°C graphical data in Figure 6-2 is band intensity data from three repeated immunoblots, normalised to lane 4, haptocorrin alone. Data is presented as means and standard deviations. Statistical analysis consisted of two-way ANOVA and Sidak's multiple comparisons test. Please note values from lane one (protein standard) were excluded from the graphical representation.

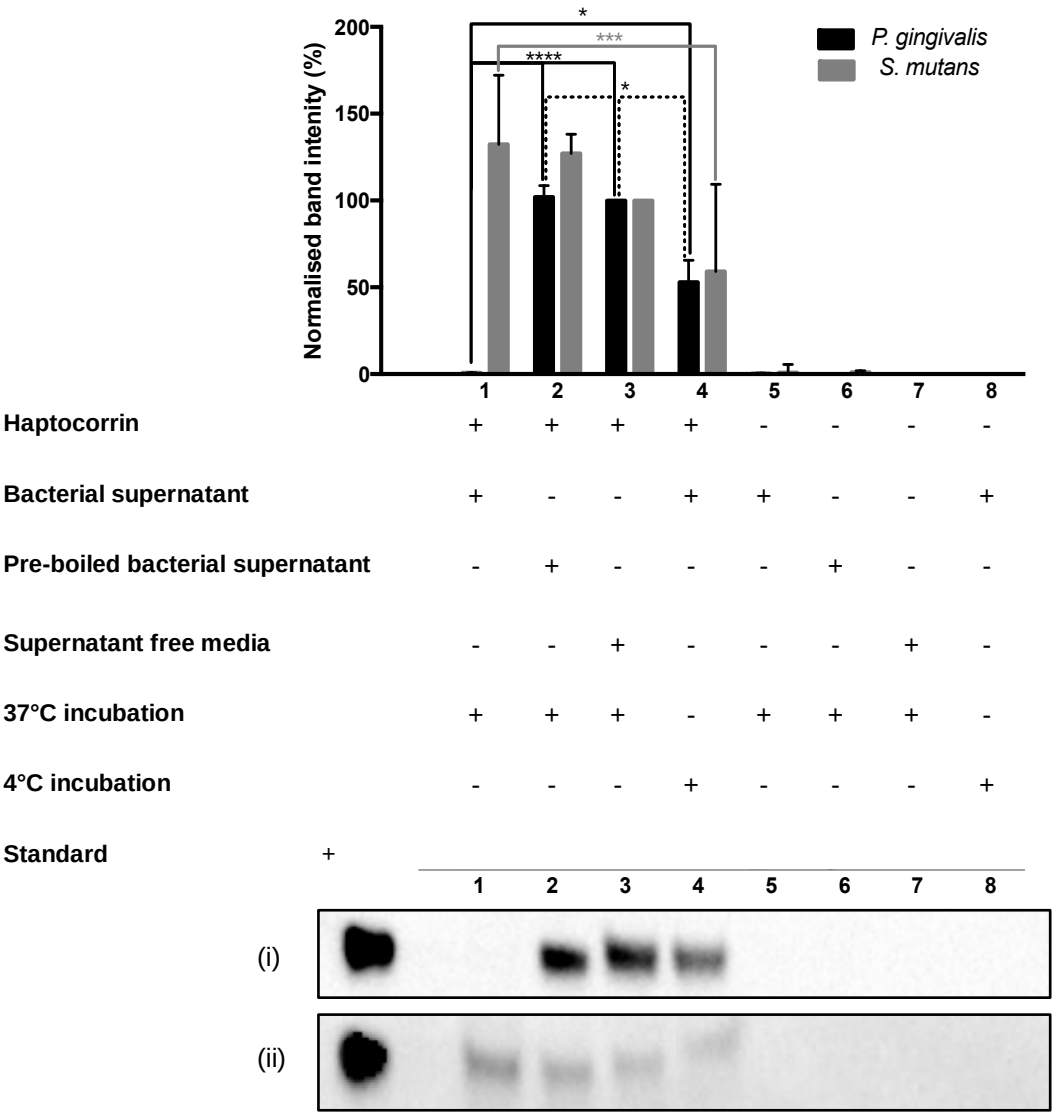

Figure 6-2 Haptocorrin incubated with *P. gingivalis* and *S. mutans* culture supernatants

Immunoblots probed for haptocorrin, representative of three repeats. Band intensity normalised to lane four, means and corresponding standard deviations are presented. (i) *P. gingivalis* strain W50 and (ii) *S. mutans* culture supernatants inoculated with recombinant haptocorrin and incubated for 1 hour under various conditions. (∗ = p <0.05), (∗∗∗ = p <0.001), (∗∗∗∗ = p <0.0001)

The influence of *P. gingivalis* culture supernatant on the band intensity of haptocorrin is titratable as demonstrated in Figure 6-3 (i), when incubated for one hour at 37°C, with reductions in culture percentage. Furthermore, a similar effect was observed when haptocorrin was incubated with resuspended *P. gingivalis* pellet. Data presented graphically are normalised to control (0% of *P. gingivalis* culture) means and standard deviations of three repeats are presented.

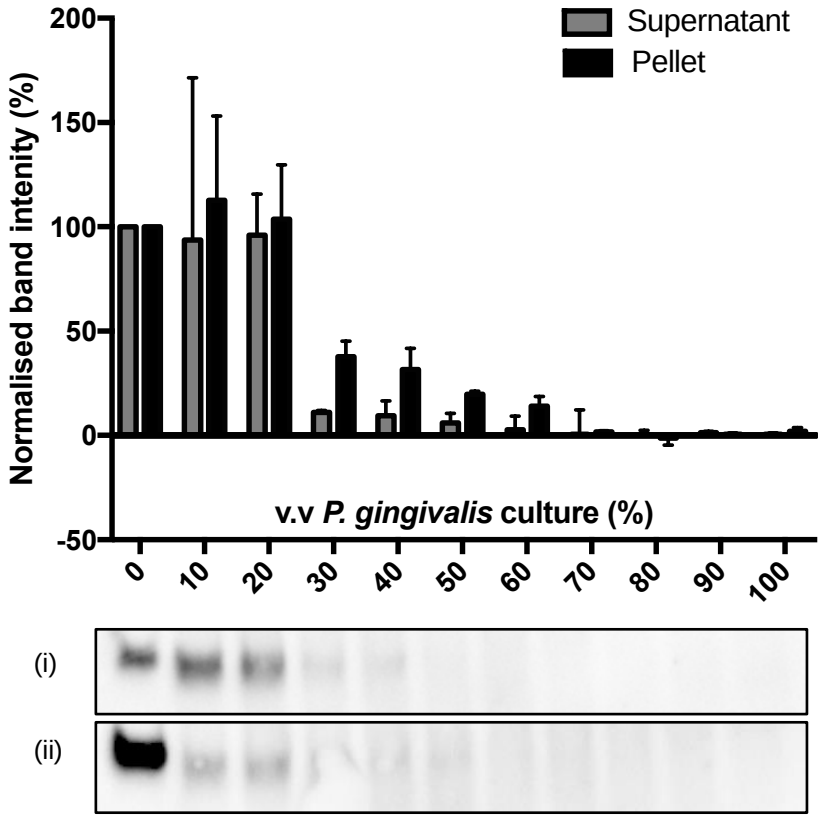

Figure 6-3 Haptocorrin incubated with titrations of *P. gingivalis* supernatant and biomass

Immunoblots probed for haptocorrin, representative of three repeats. Band intensity normalised to 0% of *P. gingivalis* culture control. Means and corresponding standard deviations are presented. *P. gingivalis* strain W50 culture separated into (i) supernatant and (ii) pellet which was resuspended prior to inoculation with recombinant haptocorrin

P. gingivalis expresses three cysteine proteases, RgpA/B and Kgp. Two knockout strains E8 (RgpA/B deletion) and K1A (Kgp deletion) were cultured as described earlier. Figure 6-4 demonstrates a significantly lower band intensity (~90%) in the wild type strain (lane 7) when compared to control (lane 3). Incubation of recombinant haptocorrin with the arginine-specific gingipain knock-out (lane 9) demonstrated a lower band intensity of ~45%, compared to the lysine-specific gingipain knock-out (K1A) which demonstrated a lower band intensity of ~75%.

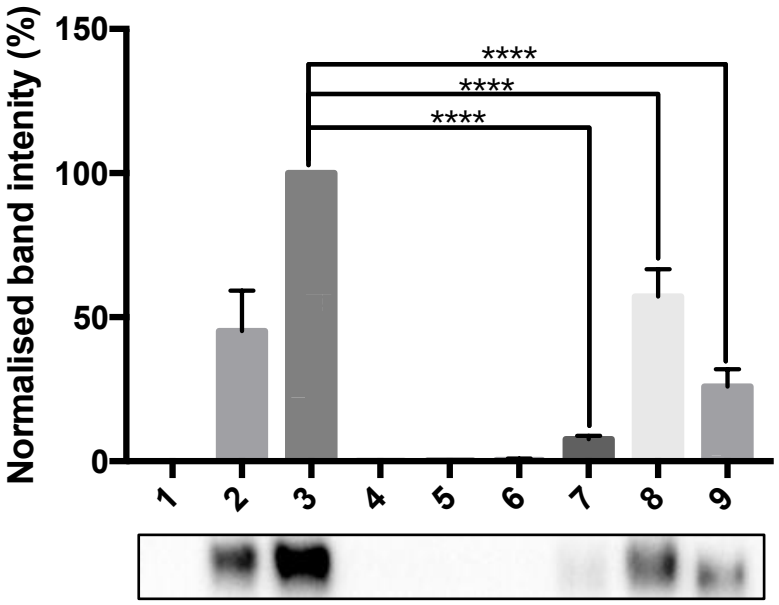

Figure 6-4 Haptocorrin incubated with *P. gingivalis* strain W50, and isogenic gingipain mutants E8 (RgpA/B), and K1A (Kgp) supernatants
Immunoblots probed for haptocorrin, representative of three repeats. Band intensity normalised to lane three, means and corresponding standard deviations are presented. Statistical analysis was undertaken by one-way ANOVA and Dunnett's multiple comparisons test. (1) Media, (2) Haptocorrin standard, (3) Media + haptocorrin, (4) W50 (wt) supernatant, (5) E8 supernatant (RgpA/B mutant), (6) K1A supernatant (Kgp mutant), (7) W50 (wt) supernatant + haptocorrin, (8) E8 supernatant + haptocorrin, (9) K1A supernatant + haptocorrin. (**** = p <0.0001)

Figure 6-5 is an immunoblot probed for haptocorrin. Saliva from two periodontally healthy individuals was incubated with culture free medium as a control (lanes 1 & 5), and culture supernatant from either wild-type W50 (lanes 2 & 6), RgpA/B mutant (lanes 3 & 7), or KgpA mutant (lanes 4 & 8). Graphically, data are presented individually as a percentage of the control band. No band was detectable post incubation with the supernatant of

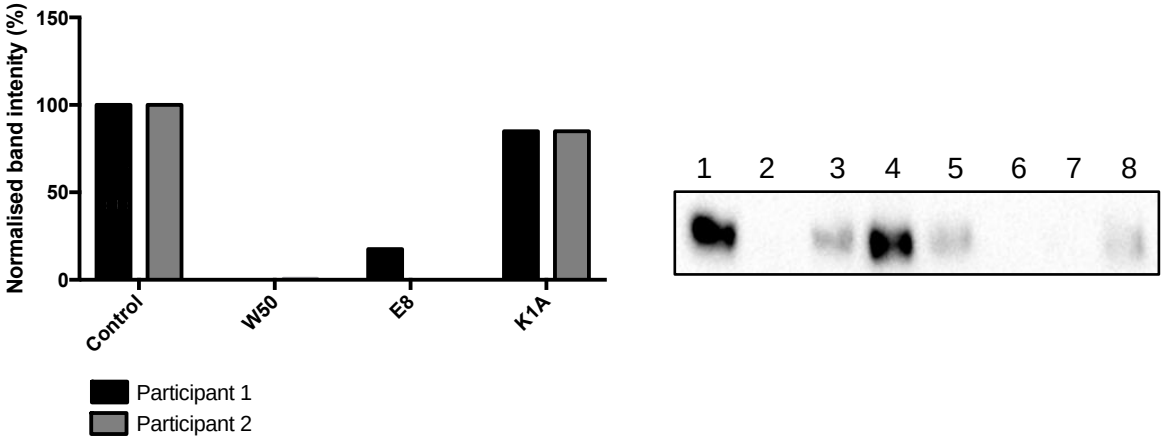

Figure 6-5 Salivary haptocorrin incubated with *P. gingivalis* strain W50, and isogenic gingipain mutants E8 (RgpA/B), and K1A (Kgp) supernatants
Immunoblot probed for haptocorrin. Band intensity normalised to unstimulated whole mouth saliva (lanes 1 & 5). (1 & 5) Whole mouth saliva, incubated with W50 (wt) (2 & 6), E8 (RgpA/B) (3 & 7), and K1A (Kgp) (4 & 8).

strain W50, band intensity was greatly reduced in both participants when incubated with strain E8, however only by ~5% when incubated with K1A.

Figure 6-6 demonstrates *P. gingivalis* growth was limited in defined medium which lacked cobalamin analogues and nutrients down stream of cobalamin dependent processes such as methionine. Supplementation of the medium with physiologically active cobalamin analogue; adenosylcobalamin, facilitates growth and was dose dependent. The positive effect of adenosylcobalamin was attenuated when the medium was further supplemented with recombinant haptocorrin.

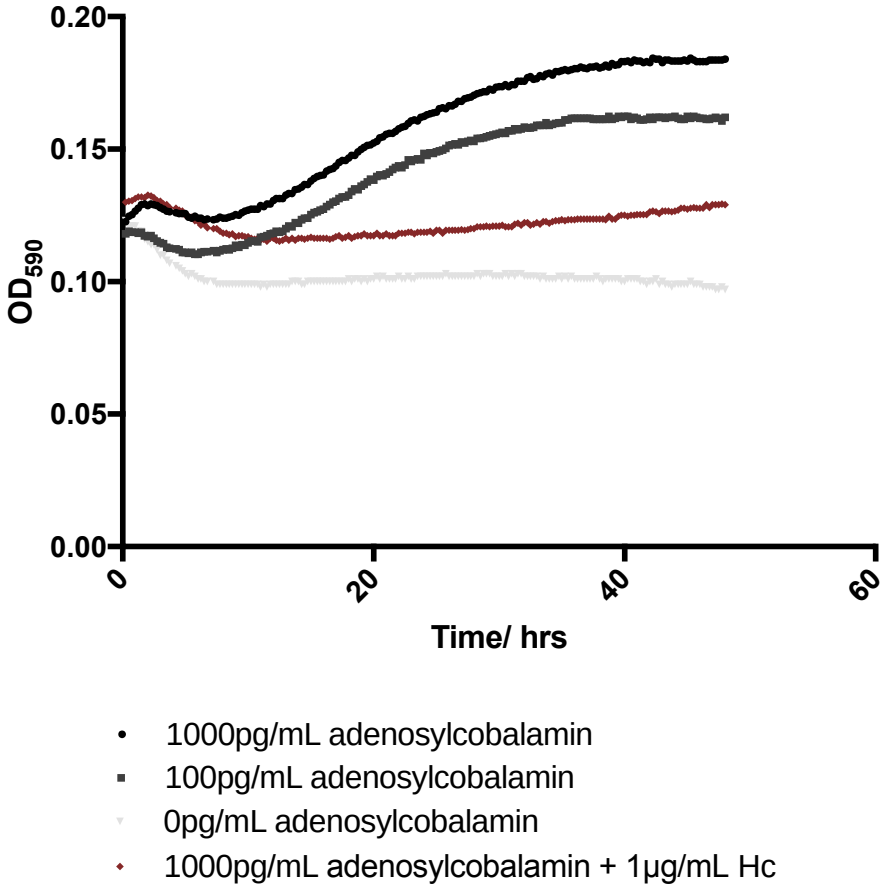

Figure 6-6 Adenosylcobalamin dependent *P. gingivalis* growth
Mean cell density of *P. gingivalis* cultured in defined medium supplemented with titrated concentrations of adenosylcobalamin and haptocorrin.

A similar positive effect on *P. gingivalis* growth was observed when repeated with methylcobalamin (Figure 6-7), another physiologically active cobalamin analogue. *P. gingivalis* growth with methylcobalamin concentration of 1000pg/mL was also limited with the addition of haptocorrin.

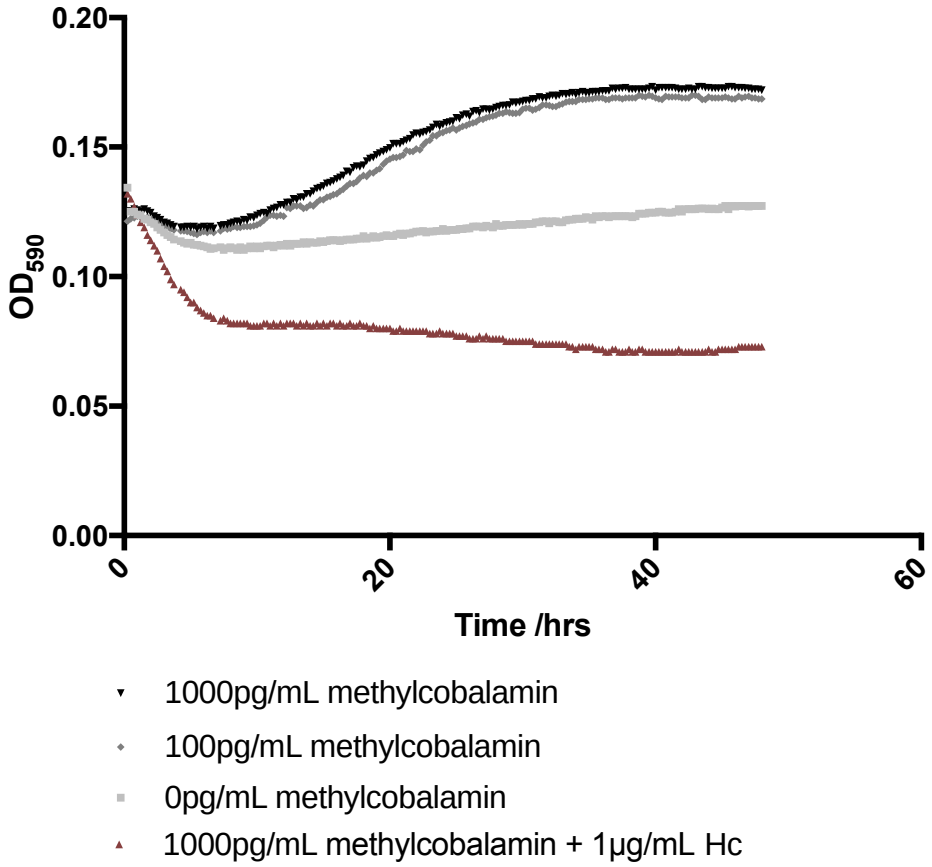

Figure 6-7 Methylcobalamin dependent *P. gingivalis* growth
Mean cell density of *P. gingivalis* cultured in defined medium supplemented with titrated concentrations of methylcobalamin and haptocorrin.

6.5 Discussion

6.5.1 Gingipain mediated degradation of haptocorrin

The preparatory work presented in this chapter suggests a secreted component of *P. gingivalis* reduces the detectability of haptocorrin by immunoblot, which does not occur when the process is repeated with *S. mutans*. Boiling of the supernatant prevented this process or incubating at 4°C reduced the severity of detection loss. The extensively researched cysteine proteases of *P. gingivalis*, gingipains, are a likely candidate for haptocorrin degradation due to their promiscuity and association with periodontal disease (273). *P. gingivalis* contains two gene loci *rgpA* and *rgpB* which encode the Arg-X specific gingipains and *kpg* which encodes the Lys-X gingipain (274). The amino acid sequence of haptocorrin was published in the late 1980s and demonstrates an abundance of arginine, and lysine residues that could be cleavage sites for gingipains (275).

In this study *P. gingivalis* strain W50 and two isogenic mutant strains E8 (Knock-out mutant for the Arg-X gingipains), and strain K1A (knock-out mutant for the Lys-X gingipain) were used (276). When assayed with recombinant haptocorrin, knock-out of the Arg-X specific gingipains had the greatest influence on reducing signal loss compared to knock-out of the Lys-X specific gingipain. Conversely, when repeated with native salivary haptocorrin, knock-out of the Lys-X gingipain had the greatest influence. Native haptocorrin is an extensively glycosylated glycoprotein with N- linked carbohydrates contributing approximately 25% of its molecular weight (277). The recombinant protein used in this study was wheat germ in origin and therefore may not contain the extensive glycosylation of the native haptocorrin (278). This difference may explain the reduced influence of the Arg-X gingipains on native salivary haptocorrin signal loss. To determine if this was a factor, deglycosylation experiments could be attempted. *In vitro* this may be performed by other bacteria within a biofilm system.

The *in vitro* assays presented in this chapter suggest gingipains, specifically the Lys-X specific gingipain of *P.* gingivalis may, at least in part, contribute to the lower concentration of salivary haptocorrin observed in the periodontal cohort presented in Chapter 5. The oral environment is far more complex, than this model system. Periodontal disease associated proteases are not only microbial in origin, host derived matrix metalloproteases (MMPs) are responsible for collagen and extracellular matrix (ECM) degradation (279). MMP concentrations are typically tightly regulated in accordance to concentrations of their inhibitors; tissue inhibitors of metalloproteases (TIMPS) (280).

6.5.2 The influence of vitamin B_{12} and haptocorrin on *P. gingivalis* growth

Biosynthesis of vitamin B_{12} is a complex ~30-enzyme-mediated step process and is restricted to a relatively small number of microbial species (281, 282). *Porphyromonas gingivalis* lacks 5- aminolaevulinic-acid dehydrogenase and porphobilinogen deaminase which are two enzymes essential for the early biosynthesis pathway; however, significant components of the later pathway are conserved (197). The data presented in this chapter suggest *P. gingivalis* growth is vitamin B_{12} dependent, the two cobalamin analogues used in this study adenosyl-, and methyl-cobalamin are the two physiologically active forms in humans. Both supported *P. gingivalis* growth in a dose dependent manner.

Haptocorrin is capable of binding a number of cobalamin analogues, which may render the vitamin inaccessible (283). The data presented in this chapter indicate that *P. gingivalis* growth was limited with the addition of haptocorrin, it is likely this was due to

sequestration of the cobalamin analogues and therefore growth was limited. Haptocorrin was not degraded by the gingipains in the latter experiments, this could be a result of reduced gingipain expression as a consequence of impaired metabolic activity, or perhaps reduced proteolysis due to a conformational change in the binding protein when bound to its ligand. Interestingly, haptocorrin is a constituent of a number of exocrine secretions including saliva, tears, and breast milk, as well as being a component of neutrophil secretory granules (91, 92). Due to the nature and localisation of haptocorrin expression, it is often suggested to facilitate an innate immune function by sequestering vitamin B_{12} and therefore limiting microbial growth, however this hypothesis is poorly substantiated in the literature (284).

6.5.3 Future considerations

To complete this work, it should be confirmed that *P. gingivalis,* and ultimately gingipains are detectible in the saliva sample of the periodontal disease cohort. A proteomics approach would also be useful to identify peptide fragments of haptocorrin in the saliva samples.

Chapter 7 General discussion

Although the functions of vitamin D-binding protein (DBP), haptocorrin (Hc), and retinol-binding protein (RBP) have been researched intensively in blood, their extra-circulatory functions have been comparatively neglected (91, 121, 285-287). All three of these proteins are present in a number of exocrine secretions, including saliva. There is a lack of literature aimed at understanding the function of these proteins within the context of saliva. The vital nature of vitamins for health, homeostasis and development drives a need for clear understanding of their assimilation pathways. The vitamin B_{12}-binding protein; haptocorrin, is considered to have an important role in chaperoning dietary vitamin B_{12} through the acidic milieu of the stomach (79). Work presented in this thesis indicates salivary vitamin-binding proteins enter the oral cavity from different routes and regulation of their expression may differ from circulatory counterparts. High concentrations of all three proteins are present at resting salivary flow and increase with mastication stimulation, therefore could play a role in dietary vitamin assimilation. Additionally, salivary vitamin binding proteins may contribute to oral homeostasis, be it either sequestration of vitamins required by the oral microbiota, or alternative functions.

7.1.1 The biomarker potential of salivary vitamin-binding proteins

The ability to non-invasively monitor systemic health biochemically is of great benefit to health professionals. Saliva offers a less-invasive, easily accessible alternative to blood (288). Attempts have been made to quantify vitamin concentration directly in saliva to limited success for vitamins D and B_{12} (289). The intention of this study was to assess the potential of salivary vitamin-binding proteins as surrogate markers of vitamin status. Data presented in Chapter 3 suggested that circulatory concentrations of DBP, Hc, and RBP displayed positive relationships to commonly applied clinical markers of vitamin D, B_{12}, and A status, respectively. However, this was not the case for salivary vitamin-binding protein concentration or outputs. The disparity in trend between vitamin-binding protein concentration across the two biofluids questions the origin of these proteins in saliva and what regulates their expression. Data presented in Chapter 5 indicated that salivary haptocorrin concentration was lower in periodontal disease patients prior to treatment and increasing significantly in those who responded to treatment. Although further work should be undertaken, haptocorrin could be suggestive of periodontal disease severity, or indeed the likelihood of a patient to respond to treatment.

7.1.2 Vitamin-binding proteins in the oral cavity, considerations for vitamin assimilation

Saliva contributes to the digestive process in a number of ways, firstly the softening of hard foods in the oral cavity, and aggregation of food particles generated by mastication. Saliva also acts to lubricate the food bolus as it transits into the stomach, preventing abrasion of the mucosal tissues (290). At a biochemical level, amylase is the most abundant salivary protein and considered to contribute to starch digestion, or at least clearance from the oral cavity post mastication (291). Salivary haptocorrin is considered to bind cobalamin reduced from the food matrix, which would most likely occur during chemical degradation of food in the stomach (214). The binding of haptocorrin to cobalamin and its analogues occurs at neutral pH, but the binding affinity increases significantly at lower pH (93). Indeed, the work presented in Chapter 4 demonstrated that all three of the binding proteins studied increased in output with mastication stimulation of salivary flow and therefore there would be an abundance in the stomach during digestion. The binding affinity of DBP for vitamin D metabolites however increases with alkalinity (292); furthermore, the lipophilic nature of vitamin D leads to incorporation into mixed micelles in the lipid fraction of a meal which may limit access by DBP (293). These factors suggest DBP does not have a role in gastric transit of vitamin D, however the gastrointestinal luminal environment neutralises in the duodenum where speculatively it could transfer to DBP. The multi-ligand receptor megalin, is expressed by the intestinal epithelium, there is evidence to suggest megalin mediates endocytosis of DBP-vitamin D complexes which could be a novel absorption route for dietary vitamin D (34). A similar argument could be made for RBP, which displays optimal binding at pH 7.4; vitamin A metabolites are also lipophilic (294).

Chapter 5 reported a lower salivary concentration of haptocorrin in a cohort of periodontal disease patients, this difference was not reflected in systemic vitamin B_{12} status, as determined by serum concentrations of total-vitamin B_{12}. This could be rationalised in a number of ways. Firstly, the samples analysed from the disease cohort were resting whole mouth saliva samples. Chapter 4 showed that stimulation of salivary flow by mastication increased salivary output of haptocorrin during digestion. This increase in output may be sufficient to maintain assimilation. Haptocorrin present in the oral cavity at resting salivary flow rate may be retained in the oral cavity for longer, and therefore open to proteolysis as demonstrated in Chapter 6. Additionally; haptocorrin is expressed by extra-oral tissues, including the gastric mucosa, which may be sufficient to maintain assimilation (91).

7.1.3 The influence of periodontal disease on vitamin status

Relationships between micronutrient deficiencies and periodontal disease have been suggested in the literature. Inadequate intake of the antioxidant vitamins (Including vitamin A) correlates significantly with periodontal disease severity (265). Work presented here demonstrated no significant difference in serum retinol status between patients or healthy controls, however there was a trend to a lower retinol concentration in the pre-treatment patient group. Both vitamin D, and B_{12} have been shown to correlate negatively with periodontal disease in the literature (55, 107). The data presented in Chapter 5 does not show a clear trend in status of either vitamin, before or after treatment for periodontal disease. Vitamin D status was no lower in the periodontal disease cohort than healthy controls. Vitamin B_{12} status was surprisingly higher in the periodontal disease cohort than healthy controls, which was inverse to the trend in salivary haptocorrin concentration. The difference in vitamin B_{12} status may be a consequence of different dietary behaviours between disease and control groups, however there is no literature to support this.

7.1.4 Is vitamin D-binding a driver of oral homeostasis?

Vitamin D-binding protein is highly multifunctional. Data presented in Chapter 3 and Chapter 4 demonstrated that DBP was highly abundant in saliva, and suggested it originated, at least partly, from gingival crevicular fluid. This supports published data which shows DBP is highly abundant in GCF (199). In addition to binding vitamin D metabolites, DBP is also a scavenger of G-actin, an activator of macrophages (by enhancing phagocytosis), and enhancing chemotactic activity of complement system components (286).

7.1.5 Haptocorrin as a potential modulator of polymicrobial community ecology

With the discovery of vitamin B_{12}-dependent riboswitches, it is becoming increasingly clear that vitamin B_{12} and its analogues are widely required by microbes for transcription regulation as well as enzymatic cofactors (295, 296). Indeed, the recently published expansion of the Human Microbiome Project highlighted the enrichment of vitamin B_{12} biosynthesis genes in the oral microbiome, the authors note the potential influence salivary haptocorrin may have on cobalamin bioavailability (297). The highly selective assimilation vitamin B_{12} assimilation pathway of humans for physiologically active cobalamin analogues is facilitated by intrinsic factor, and 80% of alternative analogues are converted to microbially-active forms by the gut microbiota (93, 298). Recent data has illustrated that riboswitches are able to discriminate between cobalamin analogues with adenosylcobalamin, methylcobalamin, and aquocobalamin acting as ligands for

different riboswitches (299). Data presented in Chapter 6 complement published work which demonstrated cobalamin (in this case methylcobalamin, and adenosylcobalamin) was exogenously required by *P. gingivalis* for growth, but also suggested that haptocorrin can inhibit *P. gingivalis* growth, presumably by sequestration of cobalamin (197). Heterogeneity of cobalamin biosynthesis in the bacterial kingdom is now considered to induce complex syntrophic relationships in polymicrobial communities, driving spatiotemporal structure (300). Numerous redundant bacterial vitamin B_{12} receptors have been identified, and are suggested to offer competitive advantage in polymicrobial communities (301). Haptocorrin is a constituent of a number of exocrine products, secreted onto mucosal surfaces, along with the iron chelating protein lactoferrin, both proteins are secreted by neutrophils (91, 226, 302). Gaining a clearer understanding of the modulatory ability of cobalamin on biofilm composition or dysbiosis may have significant clinical applications for periodontal disease.

7.1.6 Summary

The first objective of this thesis was to understand the relationship between salivary, and serum vitamin-binding protein concentrations with their corresponding vitamin ligands. The work presented in Chapter 3 showed that the concentrations of vitamin-binding proteins in saliva did not correlate with vitamin status. Furthermore, the degree of correlation between concentrations of vitamin-binding proteins across the two biofluids (saliva and serum) was greatly different between proteins, suggesting sources of the proteins in saliva varied.

The second objective of this thesis was to gain a clearer understanding of the origins of the three vitamin-binding proteins in saliva which was presented in Chapter 4. The salivary concentrations of the three proteins varied between protein and indeed between individual when salivary flow was stimulated by mastication, this suggested that the sources varied. Inter-personal variation may suggest that oral health, possibly gingival health influences the salivary concentration of vitamin-binding proteins. All three of the proteins increased in output when salivary flow was upregulated by mastication, and therefore would be present during the oral processing of food. Saliva secretions were collected from the parotid glands, non-parotid glandular secretion, as well as whole mouth, these data suggested that vitamin D-binding protein was largely non-parotid in origin, and possibly a contaminant from the gingival crevicular fluid. Haptocorrin and retinol-binding protein were detectable in all salivary types and may be expressed by salivary gland ductal cells.

Periodontal disease is associated with a number of micronutrient deficiencies, the most commonly reported being vitamin D deficiency (62, 247, 303). The objective of Chapter 5 was to assess the influence of periodontal disease on salivary vitamin-binding protein concentration, and ultimately vitamin status. Interestingly, vitamin D and A status did not differ in a periodontal disease patient cohort after standard treatment and were not significantly different from a healthy cohort. No difference was identified in retinol-binding protein concentration before or after treatment, or when compared to a health cohort. Vitamin D-binding protein concentration was lower in the disease cohort after treatment, this suggested that the positive effect of treatment on the periodontium led to reduced contamination of gingival crevicular fluid which has a high concentration of DBP. Salivary haptocorrin was higher in concentration post treatment, and concentrations of haptocorrin were significantly lower in both disease cohort samples when compared to healthy controls. Immunoblot provided evidence for degradation of haptocorrin prior to treatment. The patients that responded well to treatment had a significant increase in haptocorrin concertation post treatment, thus suggesting active periodontal disease reduces salivary haptocorrin concentration. Conversely, vitamin B_{12} status behaved inversely to haptocorrin, in being significantly higher in the disease cohort compared to controls.

The final aim of this thesis was to gain a clearer understand the interactions between salivary haptocorrin, and *P. gingivalis*. Data presented in Chapter 6 suggested that *P. gingivalis* degraded haptocorrin *in vitro*, which could, at least in part, be attributed to the secreted cysteine gingipain proteases. As with many microbial species, *P. gingivalis* required exogenous vitamin B_{12} to metabolically function (197). Chapter 6 demonstrated *P. gingivalis* growth was dependent on analogues of vitamin B_{12} being present within the growth media, and this growth was attenuated when the media was supplemented with haptocorrin. It is possible that *P. gingivalis* was not capable of secreting gingipains when metabolically limited, and therefore haptocorrin expressed a bacteriostatic effect on the bacterium.

7.1.7 Future work

The work presented in this thesis could be continued in a number of ways. Firstly, more work could be done to understand the suitability of salivary haptocorrin concentration to be used as a prognostic biomarker for the outcome of periodontal disease treatment. This would require a greater sample size of longitudinal data.

Vitamin D-binding protein is present at high concentrations in serum, saliva and particularly, gingival crevicular fluid (199). The multifunctionality of this protein is becoming increasingly clear, and it exerts influence in a number of ways beyond transport of vitamin D metabolites (286). Some of these functions, such as enhancement of C5a mediated leukocyte chemotaxis, and actin monomer sequestration, could support homeostasis of the periodontium (40, 260, 261). The high DBP concentration in GCF, and the diverse functions make it an attractive target for future work in this regard.

The data presented in this thesis compliment a number of publications which suggest the vitamin B_{12} can modulate microbial growth, and untimely polymicrobial community structure. A potentially interesting avenue of future work would be to gain a greater understanding of microbial syntrophy in relation to analogues of vitamin B_{12} and the influence haptocorrin has on this process.

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Appendices

Appendix 1 Raw participant demographic and salivary flow rate data (Chapter 3).

Participant ID	Gender	Age	Pre-weight (g)	Post-weight (g)	Duration (minutes)	Flow rate (g/min)
1	F	26	15.47	20.36	10	0.49
2	M	21	15.46	21.71	10	0.63
3	F	28	15.78	23.60	10	0.78
4	M	41	15.51	20.30	10	0.48
5	M	25	15.30	22.40	10	0.71
6	M	26	15.04	18.46	10	0.34
7	F	26	15.41	19.43	10	0.40
8	F	34	15.04	20.28	10	0.52
9	M	23	15.70	18.41	10	0.27
10	F	34	15.70	21.39	10	0.57
11	M	19	15.21	18.73	10	0.35
12	M	43	15.53	19.10	10	0.36
13	F	22	15.31	16.48	10	0.12
14	M	56	15.26	19.05	10	0.38

Appendix 2 Raw serum 25-OH-D and salivary vitamin D-binding protein concentration and output data (Chapter 3)

Participant ID	Serum [25-OH-D] (nmol/L)	Salivary [DBP] (ng/mL)	Salivary DBP output (ng/min)	Serum [DBP] (µg/mL)
1	46	155.21	75.84	273.62
2	23	99.97	62.44	236.95
3	68	674.57	527.15	631.71
4	20	331.24	158.55	443.30
5	55	165.84	117.67	456.16
6	79	186.63	63.83	443.94
7	24	116.32	46.76	299.97
8	46	141.71	74.26	331.87
9	68	181.56	49.20	250.29
10	49	866.16	492.84	380.92
11	10	41.71	14.68	153.62
12	36	420.60	150.16	327.11
13	7	140.13	16.39	341.40
14	35	250.29	94.86	261.87

Appendix 3 Raw serum total-vitamin B_{12} and salivary haptocorrin concentration and output data (Chapter 3)

Participant ID	Serum [total- B_{12}] (ng/L)	Salivary [Hc] (pg/mL)	Salivary Hc output (pg/min)	Serum [Hc] (pg/mL)
1	164	19.34	51.51	252.51
2	334	11.83	15.95	110.12
3	157	76.83	98.66	1343.04
4	163	34.53	38.82	540.77
5	228	37.65	40.30	599.81
6	347	25.93	30.11	377.54
7	350	19.89	89.85	262.93
8	194	20.07	53.42	266.40
9	195	10.00	14.45	75.39
10	316	34.72	111.36	544.24
11	189	42.77	33.44	697.05
12	326	52.48	16.96	881.12
13	298	37.83	11.52	603.28
14	454	19.89	83.39	262.93

Appendix 4 Raw serum retinol and salivary retinol-binding protein concentration and output data (Chapter 3)

Participant ID	Serum [retinol] (µmol/L)	Salivary [RBP] (µg/mL)	Salivary RBP output (µg/min)	Serum [RBP] (µg/mL)
1	2.10	1.50	0.73	98.40
2	1.44	1.67	1.04	80.19
3	2.10	1.44	0.35	88.88
4	2.26	1.56	1.22	97.56
5	2.19	1.65	0.79	93.88
6	1.89	1.65	1.17	79.77
7	1.52	1.64	0.56	92.42
8	2.07	1.60	0.64	97.08
9	2.69	1.51	0.79	103.12
10	2.54	1.54	0.42	99.16
11	1.95	1.64	0.93	82.55
12	2.68	0.84	0.30	102.99
13	1.63	0.95	0.34	95.06
14	1.99	1.02	0.12	85.75

Appendix 5 Influence of age on serum vitamin concentration and serum/ salivary vitamin-binding protein concentration and outputs (Chapter 3)

	R^2	p
Resting salivary flow rate (mL/min)	<0.01	0.99
Serum [25-OH-D] (nmol/L)	<0.01	0.96
Salivary [DBP] (ng/mL)	<0.01	0.81
Salivary DBP output (ng/min)	0.04	0.50
Serum [DBP] (µg/mL)	0.01	0.75
Serum [total-B_{12}] (ng/L)	0.17	0.15
Salivary [Hc] (pg/mL)	<0.01	0.86
Salivary Hc output (pg/min)	0.11	0.25
Serum [Hc] (pg/mL)	<0.01	0.87
Serum [retinol] (µmol/L)	0.01	0.74
Salivary [RBP] (µg/mL)	0.61	<0.01
Salivary RBP output (µg/min)	0.44	0.01
Serum [RBP] (µg/mL)	<0.01	0.99

Appendix 6 Raw participant demographic, salivary flow rate and total protein data (Chapter 4)

Participant ID	Gender	Age	Sample type	Pre-weight (g)	Post-weight (g)	Duration (minutes)	Flow rate (g/min)	[Total protein] (mg/mL)
1	F	30	Resting	15.16	19.72	5	0.33	1.32
			Chew stimulated	15.16	31.78	5	0.33	1.31
2	M	27	Resting	15.13	17.75	5	0.33	2.19
			Chew stimulated	14.57	18.41	5	0.34	1.53
3	M	28	Resting	14.98	19.44	5	0.33	1.36
			Chew stimulated	15.09	22.69	5	0.33	1.23
4	M	25	Resting	15.19	18.67	5	0.33	1.43
			Chew stimulated	14.92	27.26	5	0.34	1.11
5	F	30	Resting	15.21	17.53	5	0.33	1.26
			Chew stimulated	15.4	19.57	5	0.32	1.26
6	F	30	Resting	14.97	21.6	5	0.33	1.18
			Chew stimulated	15.18	27.91	5	0.33	1.12
7	F	32	Resting	15.26	18.67	5	0.33	1.25
			Chew stimulated	15.2	24.24	5	0.33	1.07
8	F	40	Resting	15.21	17.873	5	0.33	1.82
			Chew stimulated	15.24	31.13	5	0.33	0.70
9	M	47	Resting	15.23	17.88	5	0.33	2.35
			Chew stimulated	15.08	20.45	5	0.33	1.99
10	M	45	Resting	15.23	17.42	5	0.33	2.45
			Chew stimulated	15.09	24.78	5	0.33	1.64

Appendix 7 Influence of gender of salivary flow rate, total protein concentration, and salivary vitamin-binding protein concentration and outputs (Chapter 4)

Female vs. male participants	p
Resting flow rate (mL/min)	0.98
Stimulated flow rate (mL/min)	0.26
Resting [total-protein] (mg/mL)	0.27
Resting total-protein output (mg/min)	0.08
Stimulated [total-protein] (mg/mL)	0.60
Stimulated total-protein output (mg/min)	0.31
Resting [DBP] (ng/mL)	0.34
Resting DBP output (ng/min)	0.55
Stimulated [DBP] (ng/mL)	0.55
Stimulated DBP output (ng/min)	0.94
Resting [Hc] (pg/mL)	0.27
Resting Hc output (pg/min)	0.90
Stimulated [Hc] (pg/mL)	0.60
Stimulated Hc output (pg/min)	0.72
Resting [RBP] (µg/mL)	0.02
Resting RBP output (µg/min)	0.68
Stimulated [RBP] (µg/mL)	0.16
Stimulated RBP output (µg/min)	0.41

Tucky's multiple comparisons test

Appendix 8 Raw salivary DBP concentration and output data (Chapter 4)

Participant ID	Salivary [DBP] (ng/mL)		Salivary DBP output (ng/min)	
	Resting	Stimulated	Resting	Stimulated
1	34.32	26.95	31.30	89.58
2	134.25	133.08	70.35	102.20
3	45.95	59.53	40.99	90.49
4	42.76	31.73	29.76	78.30
5	51.15	106.76	23.73	89.04
6	24.62	19.73	32.64	50.24
7	42.23	26.63	28.80	48.15
8	94.98	39.16	50.59	124.44
9	83.73	70.36	44.38	75.57
10	92.33	38.09	40.44	73.83

Appendix 9 Raw salivary Hc concentration and output data (Chapter 4)

Participant ID	Salivary [Hc] (pg/mL)		Salivary Hc output (pg/min)	
	Resting	Stimulated	Resting	Stimulated
1	46.41	30.01	42.33	99.74
2	54.77	44.70	28.70	34.33
3	48.05	35.97	42.86	54.68
4	32.47	13.97	22.60	34.49
5	40.52	21.64	18.80	18.05
6	46.94	43.21	62.24	110.00
7	35.75	28.52	24.38	51.56
8	40.82	4.20	21.74	13.36
9	62.89	28.59	33.33	30.71
10	70.72	39.78	30.98	77.09

Appendix 10 Raw salivary RBP concentration and output data (Chapter 4)

Participant ID	Salivary [RBP] (µg/mL)		Salivary RBP output (µg/min)	
	Resting	Stimulated	Resting	Stimulated
1	1.35	1.45	1.23	4.81
2	4.61	3.30	2.42	2.53
3	1.44	2.02	1.28	3.07
4	3.41	1.45	2.37	3.59
5	1.38	2.75	0.64	2.30
6	1.82	0.37	2.41	0.93
7	0.07	0.19	0.05	0.35
8	1.38	0.72	0.74	2.29
9	1.55	0.86	0.82	0.92
10	3.78	3.06	1.66	5.94

Appendix 11 Raw salivary flow rate and vitamin-binding protein concentration data (Chapter 4)

	Participant A		Participant B	
	Resting	**Stimulated**	**Resting**	**Stimulated**
Salivary flow rate (mL/min)				
Whole mouth	0.51	2.60	1.10	2.57
Right Parotid	0.06	0.58	0.09	0.64
Left Parotid	0.06	1.07	0.11	0.46
Whole mouth (non-parotid)	0.22	0.60	0.51	1.11
Salivary [total-protein] (mg/mL)				
Whole mouth	2.09	1.08	1.72	1.45
Right Parotid	3.26	1.28	3.03	1.76
Left Parotid	3.29	0.52	2.75	1.90
Whole mouth (non-parotid)	1.00	0.90	1.09	0.99
Salivary [DBP] (ng/mL)				
Whole mouth	190.31	109.77	135.49	158.15
Right Parotid	54.37	17.83	116.64	33.18
Left Parotid	55.54	18.56	84.34	28.06
Whole mouth (non-parotid)	59.78	164.73	160.93	367.46
Salivary [Hc] (pg/mL)				
Whole mouth	5.80	4.22	7.48	3.27
Right Parotid	6.05	3.48	10.79	4.46
Left Parotid	7.04	3.06	10.54	3.76
Whole mouth (non-parotid)	1.12	4.45	5.59	3.17
Salivary [Hc] (pg/mL)				
Whole mouth	2.52	2.75	1.21	2.19
Right Parotid	1.09	6.88	1.50	0.41
Left Parotid	1.30	0.27	1.33	0.26
Whole mouth (non-parotid)	0.91	2.85	1.46	2.23

To calculate rate of analyte output, multiply the analyte concentration by salivary flow rate

Appendix 12 Raw patient demographic and smoking status data for a periodontal disease cohort (Chapter 5)

Participant	Gender	Age	Smoking status*	Response to treatment*[2]
1	M	40	N	Y
2	F	58	Y	Y
3	F	52	Never	Y
4	F	43	Y	Y
5	M	52	Never	Y
6	F	42	Never	Y
7	F	38	N	Y
8	M	42	Never	Y
9	M	57	N	Y
10	M	38	Never	Y
11	F	50	N	N
12	F	35	Never	Y
13	F	37	N	Y
14	F	39	Y	N
15	M	60	N	N
16	F	42	N	N
17	F	55	N	Y
18	F	38	Y	N
19	M	51	Never	Y
20	F	41	Never	Y
21	M	23	Never	Y
22	M	55	N	Y
23	F	50	Y	N
24	F	36	Y	Y
25	F	50	Never	N
26	M	35	Y	Y
27	M	30	Y	N
28	M	49	Never	Y
29	F	37	Never	Y
30	M	49	N	Y

* Smoking status Y = current smoker, N = previous smoker, never = never smoked

*[2] Responsiveness to treatment (as defined in 5.3.3) Y – responsive, N – non-responsive

Appendix 13 Raw serum vitamin concentrations for a periodontal disease patient cohort (Chapter 5)

Participant	Serum [25-OH-D] (nmol/L)		Serum [total-B12] (ng/L)		Serum [retinol] (μmol/L)	
	Before	After	Before	After	Before	After
1	20	20	338	339	-	-
2	44	26	319	397	2.40	1.65
3	29	20	408	410	-	-
4	46	37	593	212	2.52	2.22
5	73	20	397	212	-	-
6	26	30	434	617	2.70	2.31
7	30	27	419	276	-	-
8	65	32	615	388	-	-
9	-	-	-	-	0.97	0.92
10	35	29	313	256	-	-
11	23	24	289	763	1.55	1.72
12	25	58	740	406	-	-
13	52	54	395	316	1.25	2.03
14	-	-	-	-	-	-
15	49	42	-	-	2.40	1.81
16	39	56	476	140	-	-
17	50	40	194	605	1.42	1.03
18	28	20	507	597	-	-
19	20	72	630	302	0.52	1.59
20	57	20	285	385	-	-
21	-	-	-	-	1.11	0.96
22	33	20	409	365	-	-
23	34	35	219	247	-	-
24	86	20	297	612	1.08	1.04
25	22	34	688	689	-	-
26	25	20	323	357	-	-
27	-	-	-	-	-	-
28	40	29	682	145	-	-
29	-	-	-	-	-	-
30	-	-	-	-	-	-

Measurements before and after treatment (– denotes missing values).

Appendix 14 Raw salivary vitamin-binding protein concentrations for a periodontal disease cohort (Chapter 5)

Participant	Salivary [DBP] (ng/mL)		Salivary [Hc] (pg/mL)		Serum [RBP] (µg/mL)	
	Before	After	Before	After	Before	After
1	90.21	80.53	26.65	40.90	5.49	5.64
2	25.26	15.24	5.51	24.12	1.80	1.68
3	205.94	224.35	8.23	64.09	4.89	9.23
4	35.96	19.91	12.11	32.69	0.87	0.69
5	108.72	46.65	8.31	12.67	1.46	1.15
6	192.35	174.81	6.72	8.25	4.54	5.47
7	213.98	113.42	4.41	3.96	3.91	3.95
8	31.49	19.57	24.12	25.53	2.87	1.65
9	423.60	113.73	7.01	25.37	4.54	1.28
10	46.06	46.65	20.74	21.43	2.32	4.38
11	45.20	44.23	27.84	26.73	1.78	0.33
12	15.66	13.88	5.66	12.20	0.88	1.61
13	24.58	11.16	50.44	56.18	1.63	1.28
14	26.23	33.75	6.41	11.91	1.91	1.96
15	38.46	12.35	14.05	41.57	2.09	2.11
16	39.18	7.68	21.21	22.85	1.60	3.83
17	64.65	24.41	34.03	28.81	1.21	1.32
18	24.32	17.02	5.72	9.54	0.67	0.75
19	14.13	15.32	6.80	18.87	5.92	5.06
20	22.88	45.63	6.19	10.14	-	-
21	22.12	17.28	5.99	5.72	-	-
22	329.16	188.84	4.29	3.81	-	-
23	101.73	109.91	7.44	14.46	-	-
24	37.48	18.21	7.64	11.05	-	-
25	1773.87	1195.64	6.25	7.74	-	-
26	1295.03	831.39	6.32	8.10	-	-
27	1766.85	1304.97	3.59	4.07	-	-
28	906.23	252.57	8.18	9.22	-	-
29	322.73	1521.29	19.49	4.50	-	-
30	114.01	1271.79	4.70	4.09	-	-

Measurements before and after treatment (– denotes missing values).

Appendix 15 Table of p values, comparing analyte concentrations before and after treatment for periodontal disease in current smokers, previous smokes, and never smokers (Chapter 5)

Cohort comparison	25-OH-D	Total-B12	Retinol	DBP	HC	RBP
(Current smoker) Before vs. (Current smoker) After	0.45	1.00	>0.99	1.00	0.84	>0.99
(Current smoker) Before vs. (Previous smoker) Before	0.97	>0.99	>0.99	0.83	0.33	0.80
(Current smoker) Before vs. (Previous smoker) After	0.93	1.00	>0.99	0.92	0.042*	0.92
(Current smoker) Before vs. (Never smoked) Before	0.99	0.55	>0.99	1.00	0.99	0.58
(Current smoker) Before vs. (Never smoked) After	0.87	>0.99	>0.99	0.99	0.56	0.21
(Current smoker) After vs. (Previous smoker) Before	0.83	1.00	>0.99	0.98	0.96	0.78
(Current smoker) After vs. (Previous smoker) After	0.91	>0.99	>0.99	1.00	0.49	0.90
(Current smoker) After vs. (Never smoked) Before	0.66	0.75	>0.99	>0.99	0.99	0.55
(Current smoker) After vs. (Never smoked) After	0.93	1.00	>0.99	>0.99	1.00	0.20
(Previous smoker) Before vs. (Previous smoker) After	>0.99	1.00	>0.99	>0.99	0.91	1.00
(Previous smoker) Before vs. (Never smoked) Before	1.00	0.38	>0.99	0.96	0.60	1.00
(Previous smoker) Before vs. (Never smoked) After	1.00	1.00	>0.99	0.97	1.00	0.77
(Previous smoker) After vs. (Never smoked) Before	1.00	0.69	>0.99	0.99	0.10	0.96
(Previous smoker) After vs. (Never smoked) After	>0.99	1.00	>0.99	0.99	0.62	0.58
(Never smoked) Before vs. (Never smoked) After	0.99	0.43	>0.99	>0.99	0.85	0.97

Tukey's multiple comparisons test adjusted p values presented. * indicates significance

Appendix 16 Composition of vitamin B_{12}-free microbial culture medium (6.3.7)

Composition (per litre):

D(+)-Glucose anhydrous 40 g

Casein hydrolysate "Vitamin-free" 15 g

L-Asparagine 200 mg

L-Cysteinium chloride 200 mg

L-Cystine 400 mg

DL-Tryptophan 400 mg

Adenine 20 mg

Guanine 20 mg

Uracil 20 mg

Xanthine 20 mg

4-Aminobenzoic acid 2 mg

L(+)-Ascorbic acid 4 g

D(+)-Biotin (vitamin H) 10 µg

Calcium D(+)-pantothenate 1 mg

Folic acid 200 µg

Nicotinic acid 2 mg

Pyridoxal hydrochloride 4 mg

Pyridoxine hydrochloride 4 mg

Pyridoxamine hydrochloride 800 µg

Riboflavin 1 mg

Thiaminium dichloride 1 mg

Potassium phosphate dibasic 1 g

Iron(II)sulfate 20 mg

Potassium phosphate monobasic 1 g

Magnesium sulfate 400 mg

Manganese(II) sulfate 20 mg

Sodium acetate anhydrous 20 g

Sodium chloride 20 mg